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Circadian Rhythms and Their Impact on Aging

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Introduction

Aging is a function of the passage of chronological time, as the organism proceeds from birth to death. This simple statement does not account for the fact that individuals present differently as their chronological age progresses, and there is substantial heterogeneity among members of a birth cohort in the time of survival. For these reasons, the concept of biological age has been advanced, in recognition of this marked individual variation. Biological age takes into account the departure of any given individual from the population average in terms of time to death as well as function ability (Kim and Jazwinski 2015). Indeed, function ability, expressed in various ways, is used as a metric of biological age.

Given these considerations, biological aging can be defined as a progressive loss of function over time, in as much as such loss is characteristic of most individuals in the population. This loss of function occurs at many levels. The so-called 'hallmarks of aging' express this loss at the molecular and cellular levels, and they include genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication (Lopez-Otin et al. 2013). Except for the last, these hallmarks are entirely cell-autonomous. Intercellular communication, on the other hand, conjures up integrated function of the organism, to which the cell-autonomous hallmarks undoubtedly also contribute. Thus, we may consider biological aging to be the reflection of the loss of integrated function. At a clinical level, loss of integrated function leads to a degradation of robustness and resilience, such that biological aging is associated with an increase in the incidence of chronic disorders and susceptibility to acute diseases. These are often called the diseases and disorders of aging.

The circadian system has a period of about 24 hours, by definition. It assures that physiological processes are performed at the appropriate time of day or night. The circadian system is composed of a central clock in the suprachiasmatic nucleus (SCN) of the brain and peripheral oscillators in individual cells and tissues that operate in an autonomous fashion using similar core molecular components (Panda et al. 2002; Reppert and Weaver 2002; Froy and Miskin 2010). The central clock responds to light and dark (photoperiod), temperature, and nutritional status

(feeding behavior). The resulting entrainment is signaled by autonomic connections or circulating hormones to the peripheral oscillators. Entrainment synchronizes central and peripheral clocks, without which the endogenous rhythms free run with a period of approximately 24 h. The circadian clock through peripheral circadian oscillators affects virtually every aspect of physiology and behavior, including metabolic activity and gene expression (Panda et al. 2002; Reppert and Weaver 2002; Froy and Miskin 2010). Expression of clock genes oscillates in almost all mammalian cells, and they regulate more than 10% of the human transcriptome (Peirson et al. 2006; Storch et al. 2002).

Forty years ago, it was shown that departure from a natural 12-h light/12-h dark cycle caused a significant decrease in longevity of the fruit fly, *Drosophila melanogaster* (Pittendrigh and Minis 1972). Recently, these observations were extended to the laboratory mouse (Dubrovsky et al. 2010; Libert et al. 2012). Furthermore, there is an inverse association between the departure of the free-running period of circadian rhythm from 24 hours and lifespan in various rodent and primate species (Wyse et al. 2010). Transplantation of fetal SCN to old rodents restores the amplitude of their rhythm and extends their lifespans (Hurd and Ralph 1998), implicating central clock dysfunction in aging. However, age-related decline in the peripheral circadian machinery also occurs (Hill et al. 2013), and it may be at least partially mitigated by enhanced central clock signaling. As in animals, there is a shift in phase and decrease in amplitude of circadian rhythms with normal aging in humans (Hofman and Swaab 2006). This impairment is associated with a decline in nocturnal melatonin peak in elderly humans, contributing to the dampening of circadian rhythms (Hardeland 2010). A strong, albeit circumstantial, case can be made for an association of changes in circadian rhythm with a decline in health and age-related disease in human aging (Hardeland 2013).

It is abundantly clear that disruption of the circadian system has dire consequences for longevity (Dubrovsky 2010; Libert et al. 2012). It also has serious implications for health (Hardeland 2013), and during the course of the lifespan, clock activity becomes less and less robust (Hofman and Swaab 2006; Hardeland 2010). Virtually, every cell in every tissue marches to the time of its own molecular circadian clock. These peripheral oscillators determine the ebb and flow of metabolic processes, and they dictate the periodicity with which tissues communicate, setting and resetting physiologic thresholds throughout the organism (Panda et al. 2002; Reppert and Weaver 2002; Froy and Miskin 2010). It is obvious that these peripheral oscillators must be closely coordinated to assure the integrated function of the organism. Interestingly, integrated function of the organism decays with age, which can be observed even at the molecular level in the form of increased transcriptional noise in aged tissue (Bahar et al. 2006).

Calorie restriction (CR), a treatment that increases lifespan in many species, entrains the central clock in the SCN (Challet et al. 2003; Challet et al. 1998; Mendoza et al. 2005). It also upregulates clock genes in several tissues, among the top three biological processes thus affected (Swindell 2008). Thus, CR affects the clock and metabolism both centrally and peripherally. The Sirt1 protein deacetylase forms a complex with the Clock acetylase. This complex regulates the activity

of clock-controlled genes through chromatin remodeling (Nakahata et al. 2008) and by acetylating/deacetylating Bmal1 and Per2 (Asher et al. 2008), which are other components of the core clock (Zheng and Sehgal 2012). There is evidence that homologs of the yeast longevity gene *SIR2* play a role in the lifespan extension afforded by CR, and the activity of the mammalian Sir2 homolog, Sirt1, promotes metabolic responses similar to those found in CR (Canto and Auwerx 2009). By most criteria, the circadian system constitutes the essence of the gene–environment interface that plays such an important role in aging.

Metabolism, and especially energy metabolism, changes markedly over the lifespan (Kim and Jazwinski 2015). Calorie restriction, which extends lifespan and promotes health span, reverses many of these changes (Westbrook et al. 2014). Signaling via insulin/IGF-1(van Heemst 2010) and PI3K/Akt/Foxo (Hay 2011) plays a key role in lifespan extension, while regulating key components of metabolism. Similarly, AMPK has dual effects on metabolism and lifespan (Onken and Driscoll 2010). TOR, the cell sensor of nutrient status, plays a crucial role in coupling protein synthesis to nutrient availability, among its several functions, and it is the most widely encountered lifespan regulating device across phylogeny (Johnson et al. 2015); and, the TOR inhibitor, rapamycin, is one of only a few drugs that actually extend lifespan and health span reproducibly (Harrison et al. 2009). Finally, Sirt1 responds to NAD availability to regulate lipid metabolism and mitochondrial biogenesis (Canto and Auwerx 2009). Activation of this protein improves various features of energy metabolism, and recently it has been shown to increase mouse longevity (Mercken et al. 2014). Sirt1 is also an accessory protein to the core clock machinery (Asher et al. 2008; Zheng and Sehgal 2012).

Mitochondria, which play a central role in energy metabolism, become dysfunctional with age (Lai et al. 2002). Removal of such compromised organelles is essential to preserve youthful function, but this quality-control, autophagic process likewise becomes deficient during aging (Chistiakov et al. 2014). This deficiency is exacerbated in certain neurodegenerative disorders associated with aging (Nixon and Yang 2012). Interestingly, TOR regulates autophagy, in addition to its direct metabolic effects (Johnson et al. 2015). Thus, we must consider mitochondrial respiratory ability, ROS production, biogenesis, and turnover, in evaluating the impact of circadian regulation of metabolism on aging. The importance of ROS as a cause of aging is somewhat controversial at present, but it is clear that ROS can cause significant damage to cell components, including DNA. Melatonin, often called the hormone of darkness, is secreted by the pineal gland on stimulation by the SCN, and it is a critical output of the circadian, central pacemaker that helps to entrain both the central and peripheral circadian oscillators throughout the body (Reiter 1991, 1994; Claustrat and Leston 2015). It is also synthesized in numerous other tissues, and it is a potent antioxidant that combats ROS (Hardeland 2015).

This brief introduction highlights some of the important ways in which the circadian system impacts the aging process. It stresses the central role the circadian system plays in the integration of physiologic functions across the organism. The chapters in this book explore these topics in great detail, and they introduce the nuances associated with these and other aspects of these relationships.

Chapter 1 (Circadian Dysregulation and Melatonin Rhythm Suppression in the Context of Aging) by Reiter et al. introduces the circadian system, its operation, and the central role of the SCN and melatonin signaling. It also outlines the broad impact of this system on physiology and pathology, especially as it relates to aging.

Chapters 2–4 amplify on the themes introduced in the first chapter. Chapter 2 (Pulmonary Diseases, a Matter of Time) by Sanchez discusses the circadian system's impact on the pathophysiology of the aging lung. This chapter presents some of the recent research in this area at the cell and molecular levels. Chapter 3 (Circadian Regulation of Bone) by Maria and Witt-Enderby focuses on the impact of the clock genes on the activity of osteoblasts and osteoclasts and how this translates to bone metabolism, so important especially during aging. Chapter 4 (Aging and the Circadian Control of the Gastrointestinal System: from the Brain to the Gut Microbiome (and back)) by Cassone et al. talks about the effects of aging on the circadian clocks of the gastrointestinal system. These authors discuss novel mechanisms of entrainment and the complicity of the gut microbiome in circadian control.

Chapters 5–12 dig more deeply into cellular and molecular mechanisms associated with circadian system aging. Chapter 5 (Circadian System and Aging in Rodent Models) by Panchenko et al. presents the changes that occur in the circadian system during rodent aging and their impact on physiology and disease. This chapter describes the use of both genetic and environmental manipulations to unravel the mechanisms involved. Chapter 6 (The Circadian System and Aging of *Drosophila*) by Giebultowicz introduces the powerful *Drosophila* genetic model organism. This chapter, like the previous one, engages both genetic and environmental interventions to tease out circadian-based mechanisms of aging. It also describes novel findings related to rhythmic gene expression during aging. Chapter 7 (Circadian Control of Mitochondrial Dynamics) by Jacobi et al. expands on the metabolic threads in the previous two chapters, with an emphasis on mitochondria. These authors discuss the involvement of the core clock gene BMAL1 in mitochondrial dynamics and in mitophagy in mice and worms and the impact of these processes on both aging and lifespan. Chapter 8 (Circadian Rhythms and Proteostasis in Aging) by Desvergne and Friguet establishes a link between the circadian system, redox homeostasis, and two other fundamental cellular processes, autophagy and proteostasis. These processes undergo age-related deterioration, as demonstrated in yet another aging model, cell senescence. Gupta and Kondratov discuss the connections between the circadian clocks and mTOR signaling in Chap. 9 (Circadian Clocks and mTOR Signaling). The clocks and mTOR together play an essential role in the sensing of nutritional status and in regulation of metabolism. They impact aging, and their disruption is associated with various pathologies in animal models. In Chap. 10 (Aging and the Biological Clock), Judge et al. draw our attention to the *Neurspora* genetic model, which has played such an important role in circadian research. These authors take a systems biology approach based on the complete knowledge of the organism's transcriptional network. They demonstrate that several different metabolic pathways link aging and the clock in a reciprocal interaction. Nohara et al. in Chap. 11 (Developing Circadian Therapeutics Against Age-related Metabolic Decline) review evidence for the intimate relationship between energy homeostasis,

aging, and the circadian clock. They build on this to describe current efforts to identify small-molecule therapeutic agents that enhance circadian and metabolic functions. Such chronotherapeutics may promote healthy aging by delaying metabolic decline. Chapter 12 (The Possible Role of Epigenetics in the Memory Impairment Elicited by Circadian Rhythm Disruption) by Deibel and McDonald closes this set of chapters by presenting epigenetics as a mechanism for generation and synchronization of circadian rhythms. They discuss how memory is affected by circadian rhythms and the potential role of epigenetics in this relationship.

Stone and Tranah discuss the impact of disruption of 24-hr activity patterns in older adults on health and mortality risk in Chap. 13 (Circadian Sleep-wake Activity Patterns During Aging). These effects include increased risk of mild cognitive impairment and dementia. The authors point to the need for studies to test interventions that regulate circadian activity rhythms on health outcomes in the elderly. One of these interventions is physical activity. This book ends with Chap. 14 (Effects of Physical Activity on Circadian Rhythms in the Elderly), in which Bessot discusses the effects of physical activity on circadian rhythms. He also presents several potential mechanisms that may be involved, pointing to new avenues of investigation.

We know a good deal about circadian rhythms and how they change during aging. There is ample evidence that disruption of these rhythms accelerates aging and shortens lifespan. Often, this disruption leads to outright disease and degeneration. The impact can be readily observed at the level of fundamental cellular processes, as well as at the physiologic level. Core clock genes through their impact on the transcriptome are clearly part of the underlying mechanism. Tantalizingly, there appear to be reciprocal interactions between the circadian system and its outputs. The circadian system is the ultimate interface of the organism with the environment. It is beginning to emerge that epigenetics resides at this interface. The impact of the loss of circadian activities during human aging is manifold, and it includes cognitive function. There is promise that relatively simple interventions, such as physical activity, can reverse this decline by reinforcing circadian activity. These simple interventions may be joined by chronotherapeutics which are already in early stages of testing.

This recitation of the accomplishments of current research in the field of chronobiology of aging points to how much more we need to learn to be able to manipulate circadian activity to enhance health in an aging population. These efforts will require a systems biology approach because the circadian system impacts so many processes. Furthermore, the core clock is not only fundamentally a feedback system, but it also is subject to feedback from its distal outputs. These challenges make future research all the more exciting. It is also reassuring that interventions, such as physical activity, that have systemic effects already show considerable promise. In sum, we expect the next few years will bring us much activity in the field of the chronobiology of aging.

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Chapter 1
Circadian Dysregulation and Melatonin Rhythm Suppression in the Context of Aging

Russel J. Reiter, Sergio A. Rosales-Corral, Dun Xian Tan, Moises Alatorre-Jimenez and Carlos Lopez

Abstract Fifty years ago, little was known of the role of the prevailing light:dark environment in terms of its impact on the circadian pathophysiology of organisms. In the intervening years the field of photoperiodic regulation of the master circadian oscillator, i.e., the suprachiasmatic nucleus (SCN), has advanced at a rapid pace. The importance of the regulatory actions of the light:dark cycle, and particularly of perturbed light:dark cycles, not only on the SCN but also on the circadian production of pineal melatonin as well as the cyclic metabolism of cells throughout the body are by no means trivial. When the regular cyclic information generated and dispensed by the SCN is dysregulated, the negative consequences in terms of cellular and organismal physiology can be dire to the extent that the rate of aging and the onset and progression of a variety of age-related diseases have now been at least provisionally linked to circadian disruption and/or melatonin suppression. While the findings are not definitive, there is certainly credible data to warrant the conclusion that regular circadian rhythms at multiple levels, including a stable day: night melatonin cycle, enhance life quality and potentially delay senescence and forestall diseases normally associated with advanced age. As a result, the prolonged health span may also predispose to a longer life span. In view of the critical role of an abnormal or unusual light environment in terms of perturbing essential circadian physiological events, serious consideration should be given to rational thought about the misuse of artificial light and the consequences thereof.

Keywords Senescence · Age-related diseases · Health span · Life span Suprachiasmatic nucleus · Slave oscillators

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1.1 Introduction

Properly-timed circadian rhythms are a *sine qua non* for optimal cellular and organismal health and for the prolongation of health span, which may also translate into an enhanced life span (Agorastos and Lindhorst 2016). In the absence of well-regulated circadian biology, the molecular physiology of cells is in a state of relative chaos, the degree of which presumably is determined in part by the severity of the dysregulation (chronodisruption) and the frequency and duration of the circadian insults. There is compelling evidence that subcellular turmoil due to any influence is associated with altered metabolic pathways that jeopardize the health of cells (Hardeland et al. 2012; Reiter et al. 2012a, b). Unhealthy cells promote less vigorous organs which surely compromise the welfare and survival of organisms. The "bottom line" is that regular circadian rhythms improve cellular physiology at the molecular level.

An estimated 15% of the genes possessed by cells relate to circadian rhythms (Scott 2015). The rhythms resulting from the expression of these genes at the cellular level have been referred to as slave oscillations given that many of them are under the influence of a more superior time-giver (Lamia et al. 2008; Golombek and Rosenstein 2010; Hardeland et al. 2012; Coelho et al. 2015; Buijs et al. 2016). While there are several factors that influence the expression of rhythms within cells, a major one is the cyclic information received from the master circadian oscillator or pacemaker, the suprachiasmatic nuclei (SCN) (Pauls et al. 2016). In mammals including the human, the activity of the SCN in turn is governed in large part by the light:dark environment as perceived by the eyes (Hughes et al. 2016). The unique and intricate molecular mechanisms whereby specific, especially blue, wavelengths of light impact distinctive intrinsically photosensitive retinal ganglion cells (*ip*RGC) in the outer layer of the mammalian retinas along with the retinohypothalamic tract which transfers this information to the SCN are well described elsewhere and are not reviewed here (Lucas et al. 2014).

Once the photoperiodic information is received by the SCN, it must convey the associated message to the slave oscillators at the cellular level, whether or not this information is accurate relative to regularly-changing daily and seasonal changes in the light:dark environment as determined by the rotation of the Earth on its axis and its journey around the sun. The SCN has two known routes by which to inform the peripheral elements about the photoperiodic state. Thus, the central oscillator apprises the more distant cells via either a neural or humoral pathway (Fig. 1.1).

Since the SCN are located near the base of the telencephalic/diencephalic interface, they are in close proximity to the "head ganglion" of the autonomic nervous system, i.e., the hypothalamus. The central pacemaker has solicited the autonomic sympathetic/parasympathetic pathways as the neural route for contacting peripheral cells. One limitation of using the autonomic nervous system is that its postganglionic fibers are restricted in terms of their peripheral distribution. To solve this problem, the SCN also selected a humoral route for communication. To achieve this, the SCN utilizing the central and peripheral sympathetic nervous system,

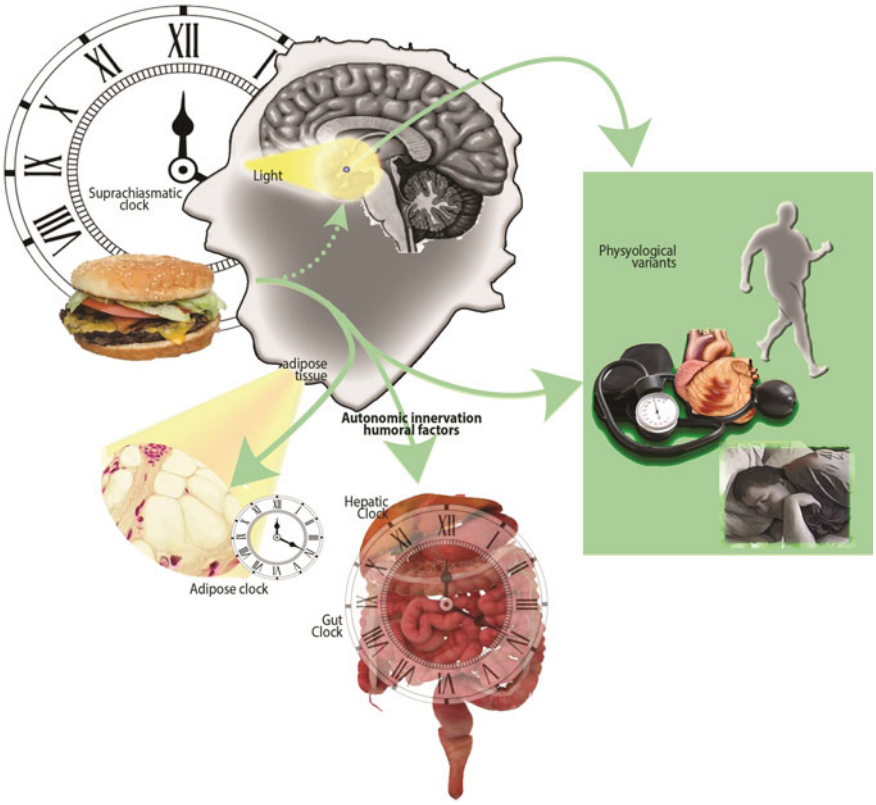

Fig. 1.1 Regulation of the master circadian oscillator (the suprachiasmatic nuclei or SCN) and its impact on peripheral cellular rhythms. The major regulatory input to the SCN comes from the light:dark cycle as perceived by melanopsin in the intrinsically photoreceptic ganglion cells of the eyes. Other factors that impact the circadian system, but to a lesser degree than the light:dark environment, include physical activity and eating. The SCN communicates its circadian information to peripheral cellular clocks via two routes, the autonomic nervous system and by means of a humoral signal, most conspicuously the melatonin cycle. Desynchronization of circadian rhythms leads to pathological disorders, represented here by the "physiological variants." Chronic disruption of the circadian system likely contributes to aging and age-related diseases

communicates with the pineal gland and dictates its metabolism (Stehle et al. 2011). In the absence of the light, the message is stimulatory at the level of the pinealocytes such that they are induced to synthesize and secrete, both into the blood (Vaughan et al. 1976) and into the cerebrospinal fluid (CSF) (Skinner and Malpaux 1999), a humoral mediator, i.e., melatonin, which contacts every cell in the organism. Any cell capable of "reading" this message, therefore, knows the status of the external light:dark environment. By utilizing both neural and humoral routes, information from the SCN is imposed on the genome of every cell in vertebrates.

With the advent of the introduction of artificial light in 1879 when the light bulb was invented and with the development of rapid transmeridian travel via airplanes, the photoperiodic information received by the SCN from the *ip*RGC is often not representative of the true daily/seasonal changes in the light:dark environment. Since the SCN is not allowed a choice in passing the altered information forward, every cell sometimes receives misinformation which disrupts their cycles and leads to circadian disturbances, i.e., chronodisruption (Erren and Reiter 2009). This contributes to malfunction of intrinsic molecular processes which create the chaos mentioned above; chaos always translates into accelerated cellular deterioration, i.e., aging. There are factors in addition to the light:dark cycle, specifically the rest/activity cycle and the feeding regimen, that also influence the function of the SCN (Fig. 1.1). This review, however, is primarily concerned with the instructions the SCN receives from the retinas and how perturbations of this information impacts cellular and organism senescence.

A major point of this report is that the aging-related consequences of chronodisruption cannot be easily distinguished from those caused by a perturbed melatonin cycle. Altered SCN rhythms are always accompanied by either a changed melatonin rhythm or a total suppression of melatonin synthesis and secretion. Given that this humoral message reaches every cell, circadian disorder becomes widespread. The problem is confounded by the fact that the plasma and CSF melatonin cycles are also designed to strengthen the central circadian clock message (Cassone 1990; Reiter et al. 2014b). In the absence of this regularly-repeating humoral message, the function of the SCN is weakened causing additional disorder which, via feedback and feedforward processes, may become a vicious cycle of gradually deteriorating molecular physiology. This leads to an acceleration of age-related processes.

The most common example of chronodisruption is that caused by light at inappropriate times, i.e., light at night. Throughout the world, well-developed societies, due to electrification, are experiencing something that organisms have never experienced during a very long period of evolution; they are experiencing what is referred to as the "end of night." It was the dependence of organisms on the regularly-repeating periods of light and darkness during evolution that was surely consequential in the evolution and the development of the SCN; after all, it was the most reliable environmental variable on which to evolve a "clock."

Light pollution is unquestionably a major factor that negatively impacts the function of the central circadian oscillator and, by necessity, the slave oscillators as well. Many studies have documented that light-at-night (LAN) changes the circadian output of the SCN, typically measured as perturbations in the blood melatonin cycle (Lewy et al. 1980) or in the urinary melatonin metabolite (Dumont et al. 2012) rhythms in humans and animals. These disturbances in the melatonin cycle have been linked to a variety of diseases/disorders that contribute to aging per se and indirectly to age-related diseases (Reiter 1995, 1997; Hardeland 2013; Hardeland et al. 2015; Opie and Lecour 2016), i.e., the promotion of pathologies. Of special note is the accelerated cancer growth, changes in glucose metabolism, skin deterioration, etc. (Kleszczynski and Fischer 2012; Haus and Smolensky 2013;

Cipolla-Neto et al. 2014) Again, the reader is reminded that any alteration in the melatonin rhythm does not occur without a corresponding change in the neural message conveyed to the periphery by the SCN. Hence, it is never possible to distinguish whether an age-related change or pathology is a specific consequence of a malfunctional melatonin rhythm or abnormal circadian neural information; most likely, they are due to a combination of these factors (Reiter et al. 2012a; Hardeland 2013, 2015).

1.2 Hallmarks of Aging: Effects of Melatonin

Aging, the rate of which varies widely among animal and plant species, is usually characterized by a time-related functional deterioration of living creatures (Jones et al. 2014). More formally, aging is defined "as a progressive loss of physiological integrity leading to impaired function and increased vulnerability to death." Historically, aging was judged on the basis of longevity in addition to the propensity of an individual to develop overt diseases. In the current era, however, aging is being investigated at the molecular level with the goal of identifying the processes that actually contribute to the aging phenotype. When these molecular processes are defined, it is the assumption that they will be modifiable and an increased healthy life span can be realized.

In a recent review, Lopez-Otin et al. (2013) suggested nine markers of aging which they feel characterize the degenerative processes at the subcellular level. While these markers are listed as being distinctly different entities, in fact, they have significant functional overlap consistent with the undoubtedly great complexity of the aging process. This entanglement will likely make identifying the specific mechanisms that underlie aging very difficult and equally problematic to treat.

A common denominator for many of the processes that are characteristics of aging is the oxidative microenvironment within cells (Forman 2016). Cells utilize oxygen as a basis of metabolism in the production of energy. Because of the relative inefficiency of the mitochondrial electron transport chain, the generation of oxygen free radicals and related non-radical oxygen-based derivatives is unavoidable (Brand 2016). This occurs when electrons are fumbled during their transfer between respiratory complexes and chemically reduce molecular oxygen to the superoxide anion radical ($O_2^{\bullet -}$). While there is a formidable antioxidant defense system, some toxic species escape detoxification and harm neighboring molecules. As a consequence, over the course of a life time, oxidatively-damaged molecules gradually accumulate which impedes efficient molecular functions. These less than proficient systems further contribute to physiological disability and the greater deterioration typical of older individuals (Sohal and Allen 1990).

Oxidative stress is certainly not the only factor that accounts for the aging phenotype; but it surely subsidizes the amount of damage molecules sustain. If these injured molecules are not repaired or removed, they become a burden to cellular physiology. Accumulated oxidative stress is mentioned as a feature of many

theories that have attempted to explain aging (Ames 1989). Melatonin, the endogenous levels of which diminish with age in most individuals, normally functions as a potent antioxidant to reduce the accumulation of oxidized molecules which accelerate senescence and aging.

Cells typically have a finite number of cell divisions they can undergo before they functionally collapse. This phenomenon was described by Hayflick several decades ago (Hayflick 1979). It is our prediction that properly-timed melatonin exposure would extend the Hayflick limit, i.e., defer cellular aging. This assumption is based on melatonin's ability to both organize the circadian biology of cells and to its actions as a multifaceted direct free radical scavenger and its indirect actions in the promotion of enzymatic antioxidative defense processes. Moreover, melatonin's high concentration in mitochondria (Venegas et al. 2012), a site where free radical formation is abundant, is consistent with the option. In this regard, melatonin has recently been designated as a mitochondria-targeted antioxidant (Reiter et al. 2016) which was proven equivalent to or more effective than synthetic antioxidants, Mito E and Mito Q, in resisting oxidative damage and inflammation. Mito E and Mito Q are industry-produced antioxidants that, because of their increased lipid solubility, concentrate in the mitochondria up to 100-500-fold (Oyewole and Birch-Machin 2015). Despite this, they are no more effective than endogenous-produced melatonin in preventing free radical-mediated molecular damage resulting from the simultaneous exposure of animals to two highly toxic bacterial molecules, lipopolysaccharide and peptidoglycan (Galley 2010; Lowes et al. 2013). Ramis et al. (2015) have recently reviewed the literature related to the relative effectiveness of synthetic antioxidants and melatonin in determining the degree of oxidative stress. Given that melatonin is so effective in curtailing the actions to toxic free radicals and the fact that its levels in both animals and many humans wane with advancing age (Scholtens et al. 2016), it seems safe to assume that the loss of melatonin including the suppression of its rhythm which contributes to circadian disruption in late life contributes to the aging phenotype which is a consequence of accumulated oxidatively-damaged molecules that occur throughout life.

More than 15 years ago, we reported that early-life pinealectomy in rats, which deprived the animals of a circadian melatonin message and dropped their circulating melatonin concentrations to barely-measurable values, caused augmented oxidative damage to all tissues in which it was measured when the rats reached 24 months of age; rats of this age are generally considered old (Reiter et al. 1999). These results likely related to the high efficiency of melatonin as a multifaceted antioxidant which, due to its low levels, allowed many mitochondrial-generated free radicals to go uncontested. This would certainly contribute to accelerated aging. These animals, however, likely had weakened circadian rhythms at the peripheral cellular level which also presumably supported the generation of an increased numbers of partially-reduced toxic oxygen derivatives. This illustrates the difficulty in distinguishing which factor, melatonin deprivation or circadian disorder, contributes most significantly to the more abundant oxidative stress in the old animals.

Some resolution to this conundrum could perhaps be gained by comparing the degree of oxidative damage in different tissues of old animals (that have been

pinealectomized at an early age). In the study of Reiter et al. (1999), for example, all tissues in which lipid peroxidation and protein carbonyls were measured (lung, liver, skin, pancreas, kidney, etc.), normally rely on the blood melatonin cycle for the majority of their circadian direction. Other tissues (e.g., salivary glands) which also received a significant amount of their circadian information via the autonomic nervous system, may exhibit less oxidative damage than cells which are not directly innervated by the sympathetic/parasympathetic fibers. These glands, since the activity of the SCN is still synchronized by the prevailing light: dark environment as perceived by the *ip*RGC, may receive regular circadian information which may better synchronize their inherent cellular rhythms thereby reducing free radical generation.

The melatonin rhythm, as a reflection of information provided by the SCN, as suggested above, likely has a significant role in directing the circadian biology of peripheral cells. The melatonin cycle, unlike the SCN-mediated circadian information conveyed via the autonomic nervous system, contacts every cell in the organism. That melatonin is in fact capable of synchronizing cellular rhythms has been documented (Jung-Hynes et al. 2010). This group showed that the intrinsic circadian rhythms of cultured cells were brought into synchrony when melatonin was added to the culture medium. This action would likely aid in slowing molecular degeneration that contributes to aging. This circadian regulatory action of melatonin may also be an explanation for the recent findings which document that melatonin sensitizes previously chemotherapeutic-resistant cancer cells to these treatments (Martin et al. 2010; Xiang et al. 2015). Given the findings of Jung-Hynes et al. (2010) relative to melatonin's ability to properly direct circadian gene expression at the peripheral level, the necessity of maintaining a regular alternating light:dark environment which propels the melatonin rhythm seems essential if aging is to be slowed. These findings are also consistent with the observations that excessive light pollution or frequent long-haul transmeridian travel, both of which cause chronodisruption and melatonin rhythm alterations/suppression, are associated with an increased risk of developing cancer and other age-related diseases. Melatonin has been commonly used by frequent long-haul travelers to combat the sleep deprivation and fatigue related to travel across multiple time zones (Cardinali et al. 2002). Besides helping to correct these disturbances, melatonin's use for this reason may also reduce the likelihood of developing age-related cancer predictably linked to frequent long-haul travel. Whether these beneficial effects of melatonin derive from is intrinsic oncostatic activity or as a result of its rhythm-synchronizing activity is difficult to determine.

1.3 Consequences of Disruption of Circadian Rhythms

During aging, there is a clear diminution in the strength of circadian rhythms which compromises organismal homeostasis in a negative way; the resulting changes contribute to morphological and physiological aging. The molecular aging

phenotype is manifested at many levels. In old individuals of any species, molecular damage comes in a variety of forms and has been studied for many years. This damage includes elevated levels of lipid peroxidation products (Grinna 1977; Zs-Nagy 1978), increased damaged protein concentrations (Steinberg and Witztum 1990; Clarke et al. 1991) and enhanced amounts of oxidized DNA (Alexander 1967; Imlag and Linn 1988). These changes also are a result of a number of factors including disease processes and perturbations of circadian rhythms, among others.

A more recently defined disorder that contributes to aging and especially to age-related diseases is the gradually-developing instability of the genome. This instability becomes greater as organisms age and is best known for its association with conditions such as progeroid-like syndromes where aging is highly accelerated and in cancer (Campisi 2005; Vijg and Suh 2013). A relationship of the deteriorating stability of the genome and cancer incidence is not unexpected since specific mutations are a requirement for a cancer to develop. While humans possess genes, e.g., SIRT1, that foster longevity (Jazwinski et al. 2010; Kim et al. 2012), the longest lived individuals often do not develop cancer; this possibly aids in their long survival (Kovacic and Somanthan 2014). SIRT1 also influences *PER2* in the master circadian clock which aids in stabilizing circadian rhythms which helps to delay/prevent disease of aging (Asher et al. 2008).

The stability of the genome declines during aging due to a variety of factors; many of these relate to an unhealthy life style (cigarette smoking, poor diet, etc.) and those that predispose to cancer (exposure to volatile carcinogens, ingestion of heavy metals, ionizing and ultraviolet radiation). A more recently defined, previously thought to be innocuous factor that aggravates genomic stability is light-at-night (LAN), i.e., circadian rhythm disturbance and/or melatonin suppression (Belancio et al. 2015). These occur especially during night shift work, late night recreational activities (so called, social jet lag), light exposure at night due to light pollution and intentional light after darkness onset when awakening. Each of these disturbs the function of the master biological clock and suppresses the melatonin synthesis/secretion cycle thereby contributing to circadian dysregulation and genomic instability (Fig. 1.2). Equally as disruptive to the circadian system are rapid changes in time zones, especially when moving in an eastwardly direction; the resulting jet lag has been linked to an increased likelihood of developing cancer (Ptacek et al. 2007), probably due to suppression of melatonin (Reiter et al. 2007; Hill et al. 2015). Genomic instability due to any reason leads to accelerated DNA damage and the consequences thereof; this includes accelerated aging.

Recently, Belancio et al. (2010a) have summarized the data that defines the role of transposable elements in genomic instability. These elements are capable of rearranging the genetic material and support for their involvement in cancer has accumulated over the last decade (Gasior et al. 2006; Belancio et al. 2010b; Lee et al. 2012; Scott et al. 2016). Genomic instability contributes to many different lesions in DNA including single base-pair substitutions or deletions and large genomic rearrangements including deletions, insertions, inversions and translocations (Belancio et al. 2010a). The large genomic changes likely contribute to the aging phenotype (Vijg and Dolle 2002; Hsieh et al. 2013).

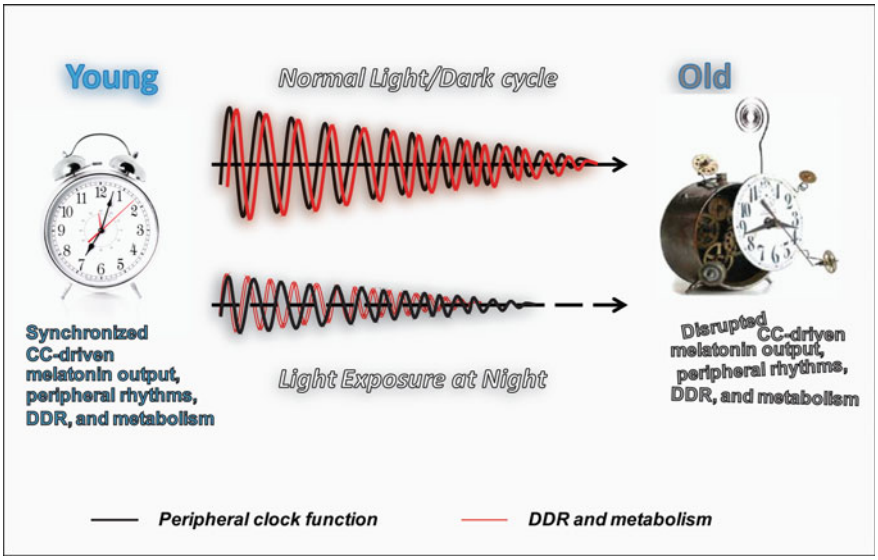

Fig. 1.2 Light exposure at night disrupts the function of the master circadian oscillator, the central clock (CC; also known as the suprachiasmatic nuclei), which results in an associated deterioration in the normally synchronized oscillations in peripheral cells. Under regularly alternating light:dark cycle, these DNA damage response (DDR) are well synchronized. The perturbed rhythms due to light-at-night negatively impacts the melatonin cycle, DDR and metabolism all of which are believed to contribute to aging and/or age-related diseases. From Belancio et al. (2015) with permission

Research has shown there are clear connections between genomic stability transposable elements (retrotransposons), circadian organization, metabolism and aging (Belancio et al. 2010a). Retro elements are mobile genetic entities that consist of two related groups, i.e., long terminal repeat (LTR) and non-LTR retrotransposons; these are represented by long (LINE) and short interspersed elements (SINE) (Belancio et al. 2008). There is now credible evidence showing that L1 activity and the circadian system are inextricably linked (deHaro et al. 2014). Hence, in an in vivo cancer model, the interaction of melatonin with its MT1 membrane receptor suppressed L1 expression; melatonin receptor had a similar effect in cultured cells. Given that melatonin is an important component of the endogenous circadian networks, it is plausible that L1 expression is under the influence of the circadian system.

As noted above, LAN is a major factor capable of disturbing the function of the master circadian oscillator, the SCN (Ikeno and Yan 2016). Since the prevailing light:dark environment drives the circadian network including the intrinsic rhythms of the slave oscillators in perhaps all mammalian cells, when darkness is interrupted by light genomic stability is also impacted as suggested by the findings of deHaro et al. (2014). The manifestations of the genomic damage that likely occurs as a result of interruption of darkness probably accounts, at least in part, for the reported

elevated cancer risk in night shift workers (Lewy et al. 1980; Reiter et al. 2007; Erren and Reiter 2008). Beyond this, LAN also accelerates the growth of already established cancer because of the suppression of melatonin (Blask et al. 2005; Hill et al. 2015). Finally, there are other metabolic consequences resulting from circadian disruption due to LAN; some of these include obesity (Cipolla-Neto et al. 2014; Coomans et al. 2015; Scott 2015) and diabetes (Ingenwerth et al. 2016) and at the cellular level depressed SIRT1 activity (Asher et al. 2008; Jung-Hynes et al. 2010) and clock gene expression (Granados-Fuentes et al. 2015). These perturbations contribute to the functional decline normally known as aging and certainly in part relate to chronodisruption- and melatonin suppression-mediated genomic instability.

1.4 Cellular Aging: The Case of Stem Cell Senescence

The ability of melatonin to forestall senescence of stem cells grown in vitro and subjected to multiple passages was recently documented. Judging from the number of approved clinical trials, it seems likely that MSC-based therapies will continue to be a common treatment paradigm in the field of regenerative medicine (Caplan and Correa 2011). The recovery of stem cells for therapy is often a limiting factor because of their numbers; this is particularly the case with certain stem cells (Jones and Wagers 2008). For example, bone marrow donation remains rather infrequent and even when marrow samples are collected there is a very low ratio of stem cells to the total number of blood forming cells. Hence, in vitro expansion of the relatively small number of stem cells that are recovered is critical to obtain the necessary number for implantation therapy. Repeated in vitro passaging of stem cells, however, introduces the likelihood of a number of disorders which compromise their usefulness, i.e., they lose their stemness and exhibit signs of senescence (Baker et al. 2015).

In an attempt to preserve bone marrow MSC (BMMSC) in a more youthful state during multiple passaging, Shuai et al. (2016) compared four low molecular weight molecules (rapamycin, resveratrol, quercetin and melatonin) in terms of preserving rat and human BMMSC in a more original state. These molecules were selected as candidates because of their previously-reported anti-aging and antioxidant actions and for their ability to enhance stem cell protection.

Shuai et al. (2016) initially showed that BMMSC lost their self-renewal potential and their osteogenic differentiation capacity during growth through 15 passages. When the four molecules (each at 10 nM) were compared for their efficacy in maintaining stemness, melatonin proved to be far superior to the other three molecules (rapamycin, resveratrol and quercetin). For example, melatonin proved highly effective in maintaining the ectopic osteogenic activity of BMMSC during long-term passaging and when the cells were transplanted into nude mice (for 8 weeks). In other models in which melatonin was tested (calvarial defect repair,

bone loss after ovariectomy, reduction of immune competence), it also had a very positive effect on the outcome measured and clearly delayed senescence.

To establish the mechanism by which melatonin functioned in their study, Shuai et al. (2016) explored the possibility that the potent antioxidant functions of the indoleamine (Manchester et al. 2015; Reiter et al. 2016) accounted for its protective actions given that cellular senescence during in vitro passaging of stem cells is known to be related to ROS generation (Busuttil et al. 2003; Parrinello et al. 2003). The incubation of BMMSC with melatonin reduced ROS formation by more than 50%; this drop was associated with a 2-fold elevation in the expression of superoxide dismutase 2 (SOD2). Likewise, the p53 pathway is stimulated by both ROS and as a result of telomere shortening limits stem cell proliferation and renewal (Bonizzi et al. 2002). When Shuai et al. (2016) examined p53 and the downstream regulator, p16, melatonin was found to significantly prevent the rise in p53 which normally would have suppressed stem cell renewal. Finally, clusters of master genes, one of which is designated NONAG, control the multipotency of stem cells by preserving them in an undifferentiated state (Tsai et al. 2012). NONAG expression is normally lost during senescence of BMMSC. Melatonin treatment preserved NONAG expression at a level equivalent to that in just-isolated stem cells. Thus, the collective results show that melatonin preserves stemness of BMMSC by reducing oxidative stress, inhibiting the p53 pathway and maintaining NONAG expression. These beneficial actions seem not to involve MT1/MT2 melatonin membrane receptors (Luchetti et al. 2014) but rather melatonin's direct scavenging actions on ROS (Tan et al. 1993; Reiter et al. 2014a) and its ability to promote antioxidant enzymes (Rodriguez et al. 2004). In the study reported by Shuai et al. (2016), treating BMMSC with luzindole (an MT1/MT2 receptor antagonist) did not interfere with melatonin's ability to safeguard these cells; thus, these receptors are not involved in anti-senescent action of melatonin.

These findings are directly applicable to aging and age-related diseases. Elevated free radical generation is commonly associated with advanced age as metabolic pathways become less efficient (Hocman 1979; Allen 1990). Simultaneously, blood melatonin levels wane as does the antioxidant capacity of this fluid (Benot et al. 1999) (Fig. 1.3). Also, early-life pinealectomy, which deprives animals of their daily melatonin rhythm and contributes to circadian dysregulation exaggerates the amount of oxidatively-damaged molecules these animals accumulate when they are 24 months of age (Reiter et al. 1999). Interestingly, the premier means of delaying aging, i.e., caloric-restriction (Meites 1990; Rae 2004), preserves molecular and cellular function and, likewise, prevents the normal reduction in pineal melatonin synthesis associated with aging (Stokkan et al. 1991). Mechanistically, the preserved melatonin production is accompanied by the retention of β-adrenergic receptors on the pinealocyte membranes; these receptors mediate the sympathetic stimulation of melatonin synthesis (Henden et al. 1992). Whether the conserved melatonin rhythm contributed to the preserved physiology of the caloric-restricted old animals has yet to be proven.

The ability of melatonin to forestall the functional capacity of stem cells is not exclusive to long-term passaged senescing BMMSC. Similarly, aging of the

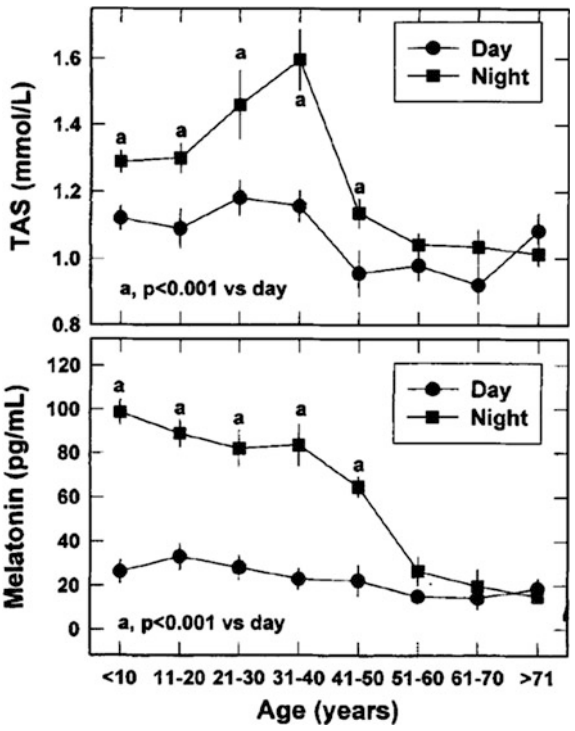

Fig. 1.3 The nocturnal levels of circulating melatonin usually drop as humans age (*bottom panel*). Associated with the nighttime reduction in melatonin is a loss of the total antioxidant status (TAS) (*upper panel*) of the blood. Thus, as humans age the reduction in the level of the antioxidant, melatonin, likely contributes to the accumulation of free radical damage to cells which hastens aging. From Benot et al. (1999) with permission

umbilical cord-derived (Lee et al. 2014) and adipose-derived stem cells (Yip et al. 2013) is also reduced when they are grown in a melatonin-containing medium. By retarding the aging of these cells, melatonin treatment also preserves their growth and physiology when they are used for transplantation therapy (Chen et al. 2014a, b; Yip et al. 2013). Finally, Zhou et al. (2015) reported that melatonin prevented MSC premature senescence when the cells were treated with the oxidizing agent, H_2O_2. This reversal was associated with an upregulation of the SIRT1 pathway, an event known to be associated with anti-aging processes (Favero et al. 2015; Ghosh and Zhou 2015). In addition to prolonging the functional half life of MSC, we had earlier predicted that culturing more differentiated cells in a melatonin-containing medium would allow them to undergo more mitoses than prescribed by the Hayflick limit (Hayflick 1979; Reiter et al. 2016).

Beyond upregulating the expression of the longevity-promoting gene, SIRT1, in MSC by melatonin, little is known about other molecular events that were changed as a consequence of melatonin treatment. Thus, while melatonin's antioxidant functions are usually used as an explanation for the enhanced functional state and prolonged survival of MSC, the circadian biology of these cells under the influence of melatonin is yet to be examined. Under in vivo situations, melatonin presumably provides stem cells with timing information as it does for terminally-differentiated elements.

Collectively, the ability of melatonin to maintain a more optimal physiological state of stem cells and reduce the rate at which they become non-functional and undergo apoptosis and aging, has applications to a number of human conditions beyond aging per se. The more rapid stabilization of dental implants, wound healing and bone fracture repair are examples where melatonin's capability to improve the functions of stem cells may come into play (Cutando et al. 2011; Clafshenkel et al. 2012; Lee et al. 2014). Even more important may be the use of melatonin to prolong the survival of stem cells when they are injected in vivo to restore tissues such as the cardiomyocytes and neurons which have no capability of regeneration. Typically, the vast majority of stem cells die when injected before they have any restorative benefit. Since the pathological conditions that require stem cell treatment are most common in the elderly, the improved function due to the use of melatonin may enhance the quality of life in older individuals. Again, in these conditions the circadian-organizing actions of melatonin should not be overlooked. Perhaps older individuals with stem cell implants should be given melatonin at a specific time of the day (night) to aid in the synchronization of the circadian rhythms of not only stem cells but other non-diseased cells as well. This may be of importance since the endogenous melatonin signal is often severely weakened in older patients because of the diminished melatonin cycle.

In vivo, stem cells are maintained in their optimal functional state by a complex signaling network (Hawkins et al. 2014). Under culture conditions due to a change in their microenvironment, these essential signals are lost; as a result researchers have sought means to defer senescence of these cells when undergoing multiple passages (Coutu et al. 2011; Eom et al. 2014). These procedures, however, have disadvantages (Guitart et al. 2010). Here, we propose that melatonin is one signaling molecule that should be tested for its ability to prolong the undifferentiated state of the cells. Other signaling molecules have already been used for this purpose (Danet et al. 2003; Lin et al. 2012) and have been shown to have only slight efficacy in this regard. Based on the comparative investigation of Shaui et al. (2016), however, melatonin should be seriously considered as an agent to delay aging of stem cells in vitro as well as after their injection. Finally, melatonin should be considered for use to prolong the survival of differentiated cells in vivo which are limited in the number of mitoses they can experience (Hayflick 1979).

1.5 Circadian Disruption and Age-Related Diseases

Epidemiological data have long suggested that circadian dysregulation due to light exposure at night (LAN) (or rapid transmeridian travel) is associated with an increased cancer risk (Hansen 2001; Erren et al. 2010; Rao et al. 2015). These findings also have strong support from experimental laboratory studies. Blask et al. (2005) and Hill et al. (2015) have repeatedly shown that LAN stimulates xenografted human tumors transplanted into immune-compromised rodents grow more aggressively; this exaggerated growth is likewise inhibited by properly-timed

melatonin administration. As noted in this survey, LAN always simultaneously disrupts the circadian melatonin cycle which also perturbs circadian rhythms more generally.

That disruption of the master clock promotes cancer growth was also recently documented in a circadian-based study reported by Papagiannakopoulos et al. (2016). Using a genetically-engineered mouse model that can be induced to develop lung adenocarcinoma, this group reported that circadian disruption due to imposed jet lag or genetic manipulation of central clock components promoted lung cancer growth and reduced the survival of the animals. Loss of the central clock components, *Per2* and *Bmal* were associated with elevated c-Myc expression, enhanced tumor cell proliferation and metabolic disruption. Melatonin levels were not measured in these animals nor was it given as a supplement to determine whether it would reverse the observed effects on exaggerated tumor growth.

The studies summarized above are only a few of the many that document that circadian disintegration and melatonin suppression may be consequential in the increased cancer risk experienced by aging individuals (Erren et al. 2016). The majority of cancers are age-related and both accurate circadian regulation and a well-preserved melatonin rhythm decrease with age. The implication is that disturbances in these two related systems contribute to cancer development and, more generally, to the degenerative changes of aging (Reiter et al. 2007).

In addition to cancer, a number of degenerative nervous system disorders are presumed to be mediated by the gradual loss of a robust melatonin cycle in the elderly; moreover, during aging the intrinsic central circadian clock is weakened and the 24-hour cycles are progressively disrupted. As an example of this, the sleep/wake cycle is often significantly advanced and sleep itself is fragmented in the elderly. The loss of adequate and restful sleep may itself be a contributing factor to aging.

Neural conditions that may be aggravated by the deteriorating melatonin cycle and a more general disturbance of the biological clock include Alzheimer disease (AD) (Pappolla et al. 1997; Hardeland 2012; Rosales-Corral et al. 2012; Ali and Kim 2015; Miller et al. 2015; Zhang et al. 2016), Parkinson disease (PD) (Mayo et al. 2005; Santos, 2012), Huntington disease (HD) (Escribano et al. 2014; van Wamelen et al. 2015), amyotrophic lateral sclerosis (ALS) (Anderson and Rodriguez 2011; Farez et al. 2016), and multiple sclerosis (MS) (Kashani et al. 2014; Wen et al. 2016). These conditions often are associated with altered circadian rhythms; whether these disturbances are a cause or an effect of these states has not been unequivocally determined. The ability of melatonin to defer the progressive development of these conditions in limited clinical trials, however, has been documented (Weishaupt et al. 2006; Medeiros et al. 2007; Cardinali et al. 2012; Lopez-Gonzalez et al. 2015). Moreover, preliminary studies with the use of melatonin to treat AD patients also suggest that this treatment may retard the progression of this devastating condition in humans (Hardeland 2016). While it is usually surmised that the antioxidant actions of melatonin account for its ability to slow the causes of AD, as noted this indole also has the capability of synchronizing the circadian system generally as well as promoting more successful sleep (Erren

et al. 2016) which may aid in forestalling CNS degeneration. Finally, melatonin may also reduce aging in the central nervous system by stimulating hippocampal progenitor cell proliferation which leads to the restoration of lost neurons in the dentate gyrus (Ethuwapranee et al. 2015).

That the rhythmic machinery underlying the sleep/wake cycle is altered becomes apparent in the older population where the timing of sleep onset shifts to an earlier time. Additionally, sleep architecture is changed since there is an increased frequency of awakenings during the nighttime sleep period along with a reduction in the total amount of non-rapid eye movement (REM) sleep (Lo et al. 2016; Mattis and Sehgal 2016). Concurrent with these changes there is often a marked attenuation of the nocturnal peak of circulating melatonin. On the other hand, there are reports of some elderly individuals who do not experience a substantial drop in maximal night melatonin values. To the knowledge of the current authors, there are no reports which examined whether the retained robust melatonin rhythm was associated with sleep architecture of younger individuals with fewer nocturnal awakenings, etc. Also, whether the well preserved melatonin cycle in a small percentage of the aged are beneficial in maintaining sleep quality seems not to have been investigated.

The differential rate of deterioration of the melatonin cycle and sleep efficiency is of special interest in light of data published by Dauchy et al. (2013) using animals. This group reported that the quality of daytime light has a major impact on the amplitude of the nighttime melatonin peak; when rats were exposed to blue wavelength-enriched light during the day, the maximal nighttime melatonin levels were increased up to ten-fold. This remarkably augmented nighttime melatonin peak enhanced the ability of the animals to resist the growth of transplanted cancer cells. Given the other actions of melatonin, the animals would be expected to have a much higher total antioxidant capacity and perhaps greater longevity. The findings also have potential implications for older individuals who may be able to improve nighttime sleep by changing their daytime light exposure to one rich in blue wavelengths.

Cardiovascular physiology also exhibits changes that have been classified as negative during aging. It is common that mean arterial blood pressure (MAP) gradually increases as individuals advance in age (Shen et al. 2015). This hypertensive response may be related to the concurrent drop in the nocturnal circulating melatonin peak and the perturbed circadian system in the elderly. In young individuals, the nighttime rise in melatonin is functionally correlated with a reduction in nocturnal systolic blood pressure (Pechanova et al. 2014). The loss of this drop in systolic pressure at night contributes to a rise in the MAP throughout life. This is consistent with what occurs in the aged. As the amplitude of the melatonin rhythm declines, the MAP rises. Hypertension is an independent risk factor for cardiovascular death and, therefore, it reduces longevity. Melatonin's benefits, besides mediating the nocturnal dip in systolic blood pressure, via its antioxidant actions likely contribute to its protection of the myocardium, as seen during cardiac ischemic/reperfusion injury (Dominguez-Rodriguez et al. 2012; Liu et al. 2015). Additionally, melatonin has been shown to exert cardiovascular

protection due to its capacity to improve the bioavailability of nitric oxide which leads to vasodilation and a drop in blood pressure (Simko et al. 2016). Thus, the loss of a stout nocturnal melatonin rise in the aged may well contribute to the elevation of MAP during late life. Elevated blood pressure invariably compromises longevity if it goes untreated.

In addition to melatonin making nitric oxide more available to provide relief from elevated MAP, its loss during aging also impacts the autonomic nervous system which may contribute to mortality. Melatonin influences sympathetic drive by enhancing GABA-ergic signaling involved in inhibiting the paraventricular nucleus by the SCN (Wang et al. 2003). Additionally, neurons in the area postrema, which are central to blood pressure regulation, are epigenetically influenced by melatonin (Irmak and Sizlan 2006). Finally, intermittent ventricular dyssynchrony has been shown to be treatable with melatonin (Dominguez-Rodriguez et al. 2016). Clearly, the melatonin rhythm has direct effects on blood pressure as well as indirect actions via its ability to modulate circadian rhythms of the autonomic nervous system (Simko et al. 2016).

1.6 Concluding Remarks and Perspectives

Even though no one has ever avoided aging and death and they are unquestionably inevitable, there is still unbounding interest in the possibility of deferring these conditions when means of delaying aging are discussed. The major consideration usually relates to prolonging life. Increasing longevity without maintaining optimal health, i.e., without extending health span, may, however, be counterproductive. On the other hand, prolonging good health into advanced age may concurrently contribute to enhanced longevity. In experimental rodents, the most reproducible means of extending life and limiting pathologies is to rather severely limit caloric intake; but the success of this procedure when applied in primates has been equivocal (Roth et al. 1999; McKiernan et al. 2011; Mattison et al. 2012). Thus, the potential benefits of reducing food intake for the purpose of stretching the life span of human remains obscure. This, however, has not dissuaded scientists from trying to identify molecules that can be consumed and that mimic the cellular molecular actions observed in underfed rodents (Ingram and Roth 2015). The amount of resources dedicated to identifying elixirs that may slow down time is currently massive and even well-established, renowned scientists are self-testing molecules that may defer age-related diseases and enhance longevity.

In the current report, we examined the data that suggests a relationship between circadian biology and the processes of aging. Circadian rhythm disturbances cannot be considered in terms of their effects without taking into account the melatonin cycle. This is a requirement since the central Zeitgeber, i.e., the SCN, and the melatonin rhythm are mutually interactive. Any disturbance of the message sent out from the SCN is always accompanied by a perturbation and often a suppression of the pineal melatonin production and release. Moreover, any alteration in melatonin

synthesis and secretion changes the function of the master clock given that melatonin in the blood, or more likely in the CSF (Reiter et al. 2014b), feedbacks on the SCN to modulate circadian rhythmicity, i.e., it serves as a chronobiotic.

The data summarized herein shows that the SCN-pineal axis may well impact the progress of senescence development and especially the likelihood of delaying the onset or severity of age-related diseases. A major difficulty with assessing the specific role of an imposed circadian disorder on cellular function stems from the fact that there is no procedure nor drug that uniquely and precisely destroys circadian biology without also seriously impacting the melatonin rhythm. While exogenously-administered melatonin has been used to potentially define the functional relevance of this molecule in altering the rate of aging and disease prevention, the evidence remains sketchy whether this relates to the chronobiotic actions of this indole or is exclusively a result of its diverse antioxidant functions. Certainly, melatonin has been successfully used experimentally to delay the onset and progression of age-related disorders; however, whether it directly defers aging per se remains unclear.

Relative to the circadian system and aging, it has been difficult to link the functional deterioration of this system specifically to the rate of aging or of age-related diseases. Rather than contributing to aging, the weakening circadian rhythms may be a consequence of aging rather than being causative. Also, while there is no doubt that the circadian system suffers dysfunction in the aged, whether this relates to specific disease phenotypes remains to be defined.

As summarized by Lopez-Otin et al. (2013), there are many facets that likely contribute to the aging phenotype and to mortality. These include genomic damage that goes unrepaired and therefore accumulates with increasing age. This issue is particularly obvious in humans suffering with progeria-related diseases where DNA damage occurs at an unprecedented rate. What are defined as epigenetic shifts also may advance age-related functional deterioration (Wu et al. 1993). These authors found that when DNA methylation in human cells is mapped over time, some areas of the genome become hypermethylated while some do not. Likewise, histone modification changes as a function of age in some tissues. It seems likely that the accumulated damage and epigenetic shifts may relate to circadian disturbances and changes in the melatonin cycle although this has yet to be determined. While the accumulated DNA damage that occurs is often a result of oxidative stress, it is probable that melatonin, because of its antioxidant properties, slows DNA damage and thereby contributes to slowing cellular aging.

Telomeres, the repetitive sequences on the ends of linear chromosomes shorten with age and contribute to the instability of the genome. DNA polymerases are incapable of replicating these chromosomal segments. As telomeres shrink, the cell undergoes senescence and implodes due to apoptosis. Agents that stimulate telomerase, which renews telomeres, delay cellular aging (Hewitt et al. 2012). Experimentally, melatonin does enhance telomerase activity in normal cells (Hardeland 2013) suggesting that this indole may contribute to preserving good health and deferring aging; however, there are no reports that definitively prove

either blunting of the melatonin cycle or disturbed circadian rhythms in the aged as being related to changes in telomerase activity.

There is a great diversity in the trajectories of the rate of aging across the tree of life and rather few species have been investigated as to why these species age differently (Promislow and Harvey 1990; Jones et al. 2008). Some rodents are relatively short lived, e.g., the rat, while others have an uncommonly long life, e.g., naked mole rat (Austad 2007). Bird species also show a similar wide diversity in their duration of survival (Barja 1998; Herrero and Barja 1998). A satisfactory explanation as to why these species differ so markedly in terms of their survival has yet to be determined.

The functional connections between either altered circadian rhythms or perturbations of the melatonin cycle and aging remain nebulous. The reader is reminded that these rhythms developed over a very long period of evolution during which the light:dark cycle was precisely reproduced from year to year as dictated by the rising and setting of the sun. Hence, severe disturbances of these precise rhythms that result from the introduction of inappropriately-timed light would be expected to have health and age-related consequences, some of which may be dire. This may also have to be taken into consideration when humans finally populate planets where the light:dark environment differs significantly from that on Earth.

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Chapter 2
Pulmonary Diseases, a Matter of Time

Cecilia G. Sanchez

Abstract Age-related progressive loss of lung function contributes to disability and premature death. How the circadian clock regulates the development and progression of age-related lung diseases, and the underlying molecular mechanisms, are still largely unknown. The chronobiology of the lung and the role of the circadian clock in the pathophysiology of age-related lung disease will be discussed in this chapter. We describe the molecular links between the circadian clock and the specific hallmarks of aging to provide a better understanding of healthy lung aging and age-related lung disease. Furthermore, the impact of external factors in the intrinsic circadian regulation will be integrated into the complex profiling of age-related lung diseases as well as chrono-therapeutic approaches. Finally, gaps in our knowledge and future directions will be discussed.

Keywords Circadian clock · Lung · Sleep · COPD · Emphysema · Pulmonary fibrosis · Aging

2.1 Introduction

The mammalian circadian system is organized in a hierarchical manner. A central pacemaker located in the suprachiasmatic nucleus of the brain's hypothalamus is responsible for the synchronization of cellular circadian oscillators in most peripheral body cells, including the cells in the lung. Furthermore, cell-autonomous oscillators control cellular functions in response to specific stimuli (Albrecht 2012; Bando et al. 2007; Reilly et al. 2007).

Circadian rhythms are generated at the cellular level by an autoregulatory feedback loop of interconnected transcription factors referred to collectively as clock genes (Hirota and Fukada 2004; Mohawk et al. 2012). In mammals, the

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BMAL1:CLOCK activator complex regulates expression of period (PER1–3) and cryptochrome (CRY1–2) genes. The heterodimers PER and CRY, are phosphorylated. Upon translocation to the nucleus, PER and CRY proteins associate with the BMAL1:CLOCK complex and suppress their own transcription by blocking the activity of the BMAL1:CLOCK complex (Grimaldi et al. 2009). Nuclear receptors REV-ERBα (NR1D1; nuclear receptor subfamily 1, group D, member 1) and retinoic acid-related orphan receptor-α (RORα) regulate the timing and amplitude of BMAL1 expression. Circadian disruption, defined as changes in amplitude or timing of clock gene expression, can alter clock-controlled output genes and, consequently, mechanical and physiological processes in the lung (Sundar et al. 2014; Dong et al. 2016b; Hadden et al. 2012). Much of the current evidence demonstrating adverse health outcomes from circadian rhythm disruption comes from studies with shift workers. Circadian rhythms synchronize the sleep-wake cycle and also play a major role in the onset and severity of diseases. A lack of synchrony between central and peripheral oscillators increases the risk of metabolic, endocrine, and cardiovascular disease. In murine studies, a single 6-h advance repeated over 8 weeks can increase mortality in older mice (Davidson et al. 2006). A daily 4-h advance for 10 weeks leads to metabolic, endocrine, and neurophysiological disease (Karatsoreos et al. 2011). Moreover, in humans, prolonged sleep restriction was shown to alter lipid and carbohydrate metabolism, insulin resistance, growth hormone and corticosteroid secretion. In consequence, it may increase the risk of obesity, diabetes, and cancer (Buxton et al. 2012; Karlsson et al. 2001; Van Cauter et al. 1997; Hennig et al. 1998; Grundy et al. 2013). Similar to shift workers, patients in the intensive care unit experience circadian rhythm disruption due to noise, interaction with caregivers, mechanical ventilation, pain, medications, artificial light, and the illness itself, accompanied by sleep fragmentation and delirium (Madrid-Navarro et al. 2015; Figueroa-Ramos et al. 2009). Internal desynchronization may also arise in response to other environmental perturbations, including feeding time, viral infection, and cigarette exposure (Evans and Davidson 2013; Hirota and Fukada 2004; Wolff et al. 2013). Nevertheless, a temporary misalignment, like jet lag, doesn't have significant consequences.

Posttranslational modifications can also affect the molecular clock regulators (Hirayama et al. 2007; Nakahata et al. 2008). Nevertheless, the changes in those modifications and their effect on clock-dependent cellular functions in the lung are largely unknown. Recent studies revealed that TGFβ1, a pro-fibrotic mediator, increases the amplitude of Bmal expression and the rhythmicity of other circadian clock regulators. Those studies also showed that TGFβ1 regulates the post-translational changes in BMAL1 and that BMAL1 promotes the effects of TGFβ1 in epithelial cells and during myofibroblast differentiation (Dong et al. 2016a). Nevertheless, there is a need to identify the impact of circadian disruption at the tissue level and the associated relevant posttranslational modifications in specific pathophysiological conditions. It is also important to determine the use of clock regulators as biomarkers of pulmonary dysfunction in specific lung pathologies, as well as the potential for novel Chrono- pharmacology agents and strategies to improve vaccine efficacy and to treat and/or prevent the progression of age-related lung disease.

2.2 Chronobiology of the Lung

The human organism shows an intricate temporal structure consisting of rhythmic variations, at multiple frequencies, of most biologic variables such as development and aging. Rhythms are also externally synchronized by environmental factors such as light-darkness, social routine, work schedule, and food intake (Smolensky et al. 1999; Haus et al. 1993). The clock modulates stress responses and physiological processes unique to each organ (Ko and Takahashi 2006; Panda et al. 2002; Stangherlin and Reddy 2013; Gossan et al. 2013; Patel et al. 2014). In consequence, circadian variations are found in respiratory volume (RV), respiratory minute volume (RMV), vital capacity (VC) and forced expiratory value per sec (FEV1) (Zaslavskaia et al. 2004). Airway caliber and inflammation also follow circadian patterns. Airway resistance peaks in the morning, reaches its lowest point at noon, and increases in the afternoon (Hruby and Butler 1975). The circadian clock also modulates the intrinsic properties of each cell at the levels of mRNA and protein expression, metabolism, and responsiveness to extracellular stimuli (Fig. 2.1). In addition, the direct exposure to high concentrations of oxygen, pollutants, particulates, smoke, and pathogens, as well as the cumulative effects of sleep disruption,

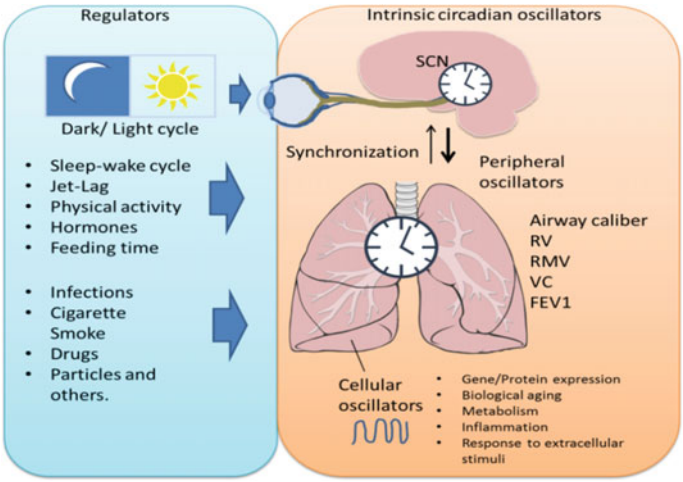

Fig. 2.1 The dark-light cycle is the most important synchronizer of the circadian rhythms. Other environmental factors include sleep–wake and activity cycles, meal timing as well as direct lung exposure to infections, drugs, cigarette smoke and particles. Intrinsic circadian oscillations in the brain (SCN) and the periphery organs. In this case, the lung is synchronized via humoral signals and the autonomic nervous system. The circadian system actively synchronizes the temporal sequence of biological functions with the environment. Airway caliber, RV, RMV, VC and FEV1 are circadian regulated. Cellular oscillators regulate mRNA/protein expression, hallmarks of biological aging, metabolism, inflammation and response to stimuli. *SCN* suprachiasmatic nuclei. Respiratory volume (*RV*), respiratory minute volume (*RMV*), vital capacity (*VC*), forced expiratory value per sec (*FEV1*)

alters the circadian clock in the lung (Gebel et al. 2006; Hwang et al. 2014; Hu et al. 2011; Sundar et al. 2014; Vasu et al. 2009; Pilorz et al. 2014) (Fig. 2.1).

In an elegant work, Haspel et al.(2014) determined that approximately 1067 genes exhibit reproducible rhythmic expression in healthy lung and that 321 of these are uniquely rhythmic in mouse lung (Haspel et al. 2014). An important observation of the authors was that immune processes are heavily represented in the circadian transcriptome of the healthy mouse lung, a finding not emphasized in published meta-analyses of other mouse solid organs (Yan et al. 2008). As a result, they concluded that the mouse lung might be a valuable tool in examining how inflammation affects organ-wide circadian regulation.

Sex-based disparities occur in sleep disorders and chronic lung diseases. Therefore, it becomes critical to determine sex-based differences in circadian regulation of the lung transcriptome in a healthy lung. It is known that gonadal steroids in both male and female rodents modify the amplitude, movement, and phase of daily rhythms (Fitzgerald and Zucker 1976; Morin et al. 1977; Albers 1981; Davis et al. 1983; Iwahana et al. 2008). Recent studies in rats revealed a direct effect of progesterone on pineal melatonin release and strongly suggest a temporal effect of this ovarian hormone on pineal function (San Martin and Touitou 2000). Therefore, we can expect that significant changes occur in the central and peripheral clocks during the menstrual cycle. In fact, Forced vital capacity (FVC) and Forced expiratory volume (FEV1) were found to change during the menstrual cycle in studies of cystic fibrosis patients; the same study found significantly higher FVC and FEV1 during the luteal phase than during ovulation and menstruation, probably due to changes in progesterone level (Johannesson et al. 2000). Besides, exposure to constant light was shown to upregulate follicle-stimulating hormone and estradiol levels and downregulate progesterone level in both the maternal and fetal circulation (Gao et al. 2016). Expression of clock genes was also studied in female, and male mice maintained on a 12 h light/12 h dark cycle (control) or exposed for four weeks to a regimen of shifting light mimicking chronic jet lag (CJL). Interestingly, among CJL females, expression of clock genes Bmal1 and Rev-erbα decreased and expression of their repressors, Per2 and Cry2, increased. In contrast, among CJL males, expression of Clock was decreased, whereas Per2 and Rev-erbα expression increased. Those changes correlated with alterations in lung mechanics, leading to the conclusion that circadian disruption alters lung mechanics and clock gene expression in a sexually dimorphic manner (Hadden et al. 2012). Future understanding of the role of neuroendocrine mediators, sex differences, and environmental factors in biological rhythms is central to advancing our understanding of age-related lung disorders.

There are rhythms of about seven days duration (circaseptan rhythms), known as the rhythmic response to an environmental load, antigen, or therapeutic agents, and poorly studied in the context of lung therapies. A study in 26 elderly patients with chronic obstructive pulmonary disease (COPD) analyzed the circadian, circaseptan, and circasemiseptan (3.5-day) rhythms of external respiratory function (ERF) before and after routine treatment (RT). In this study, Circasemiseptan variations were found in respiratory volume (RV), vital capacity (VC), and FEV1

before RT. After RT, these were detected in respiration rate (RR), RMV, and VC (Zaslavskaia et al. 2004). Circaseptan rhythms were also studied in an animal model of urethane-induced lung cancer. In this model, powerful high-amplitude of circaseptan oscillations arose after injection of the carcinogen in the targeted lungs (Riabykh et al. 1994).

Rhythms of approximate 1-year duration are called circannual rhythms, this type of rhythm is observed experimentally in animals kept under experimental laboratory conditions and never exposed to variations in day length or temperature. Seasonal variation observed in the immune response may be due to environmental factors including differences in day length or in the function of the pineal gland (light/dark-related) or the thyroid gland (temperature-related) and the prolactin levels. Also, differences in exposure to drugs and a variety of antigens, including microorganisms can induce seasonal variation. Various biological processes with immunological roles show seasonal variation in humans. Nevertheless, how seasons might broadly impact human pathophysiology is still unknown (Dopico et al. 2015).

Importantly, seasonal variations were found in 9 of the 16 clock genes, BMAL1, CLOCK, CRY1, CSNK1D, CSNK1E, NR1D2, RORA, TIMELESS30, and NFIL3. BMAL1 was found to be a common seasonal gene with increased summer expression in each peripheral blood mononuclear cell (PBMC) data set. Interestingly, the glucocorticoid receptor (NR3C1), which has anti-inflammatory properties, showed a strong positive correlation with BMAL1 expression, with lowest expression level in winter. By contrast, receptors for the prostaglandins (PTGDR, PTGIR, and PTGER4), leukotrienes (CYSLTR1), and oxoeicosanoids (OXER1) were expressed at a higher level in the winter. Receptors for adiponectin (ADIPOR1), estradiol (ESR2), and antidiuretic hormone (CUL5) were expressed at a higher level in the summer (Dopico et al. 2015). This study also revealed that 5136 unique genes out of 22,822 genes tested showed significant seasonal differences in expression (Dopico et al. 2015). Therefore, seasonal changes in expression of circadian genes might impact the immune responses. In fact, the circadian rhythms influence the immune functions of natural killer cell activity, lymphocyte proliferation, monocyte and macrophage gene expression, as well as the levels of inflammatory mediators, IL-6, tumor necrosis factor and IFN-γ (Boivin et al. 2003; Thompson et al. 2014; Hwang et al. 2014; Martin 1999).

2.3 A Time for Pulmonary Diseases

The lung is constantly responding to insults during chronological aging. Consequently, older individuals have an increased risk of developing respiratory impairment. Dysfunction in the molecular clock mediates changes in the immune, inflammatory, and DNA-damage responses that correlate with exacerbation of some

pathological respiratory conditions, such as pulmonary edema, asthma, and allergic attacks (Froy 2010). Nevertheless, the effects of environmental exposures on circadian-clock regulation and the impact of circadian disruption on various pathophysiological lung conditions are not completely understood. Here, we will review some of the literature concerning abnormalities in the circadian clock and their association with lung disease. We expect that research in the next decade will determine the concurrence of rhythm-related risk factors leading to pathologic events and the development of chronic lung diseases. We also hope that the study of the circadian regulation will provide a better understanding of the onset and progression of those diseases and identify effective treatments. Furthermore, we expect that future research will also result in the use of clock molecules as biomarkers of pulmonary dysfunction.

2.3.1 Chronic Obstructive Pulmonary Disease (COPD)

COPD is characterized by the loss of lung elasticity due to emphysema and airway obstruction as a result of inflammatory narrowing and fibrosis. At the cellular level, COPD represents a complex interplay between lung epithelial cells, endothelial cells, neutrophils, macrophages, and multiple subpopulations of both $CD8^+$ and $CD4^+$ T cells (Bosken et al. 1992; Lams et al. 1998; Saetta et al. 1999; Amin et al. 2003; Lee et al. 2011). In spite of recent advances, there is still a fundamental lack of knowledge about the cellular, molecular, epigenetic, and genetic causes of COPD (Sanchez 2016a). Considering that smoking tobacco alters major components of the circadian clock and constitutes a major risk factor for COPD, an understanding of the role of the circadian clock in the pathogenesis of COPD is a critical need (Vestbo et al. 2013; Mannino 2003). Cigarette smoke (CS) can affect circadian secretion of corticosterone (CORT), an adrenal steroid and a major component of the stress response, and serotonin (5-hydroxytryptamine; 5-HT), leading to sleep and mood disorders in smokers and patients with COPD (Sundar et al. 2014).

In patients with COPD, airway obstruction progresses with time and exacerbations of the disease tend to arise about once per year (Tashkin 2013). Patients with COPD experience sleep abnormalities, including symptoms of insomnia, daytime sleepiness, and nocturnal oxygen desaturation due to hypoventilation. These defects were determined in early studies on sleep quality using overnight polysomnography (Fleetham et al. 1982; Cormick et al. 1986). The studies with morning-symptom diaries confirm that morning is the most difficult time of day for many patients with COPD (Globe et al. 2016). Sleep disruption also causes significant neuropsychological changes, and its impact is perceived in the quality of life of the patients with diverse chronic lung diseases (Lewis 2001). The severity of the symptoms during COPD exacerbations varies diurnally and shows elevated risk for intubation during early morning hours (Tsai et al. 2007; Truong et al. 2016).

Smoking is the greatest risk factor for COPD (Vestbo et al. 2013; Mannino 2003). Exciting research is emerging showing the effects of tobacco smoking on the components of the circadian clock. Gene expression analysis of the lung from rats exposed to CS revealed a CS–exposure-dependent shift in the cyclical expression of genes involved in the inflammatory response and those controlling the circadian rhythm. (Gebel et al. 2006). Another study using whole CS (WS) and filtered CS (FS) revealed a differential CS-mediated modulation of genes related to circadian pathways in the lung. For example, Rev-Erb alpha expression is downregulated by WS and FS compared to controls. Interestingly, WS was more efficient than FS in decreasing Rev-Erb alpha expression (Vasu et al. 2009).

At the posttranslational level, it was found that Bmal1 is preferentially acetylated and degraded in the lungs of mice exposed to CS and in patients with COPD, compared with lungs of the nonsmoking controls, linking it mechanistically to CS-induced reduction in Sirt1. In fact, the targeted deletion of Bmal1 in lung epithelium confirmed an increase in CS-mediated inflammation (Hwang et al. 2014).

Lung function in COPD patients is also altered following viral infection, including infection with influenza A. Chronic CS exposure combined with infections with influenza A, changed the timing of clock gene expression in parallel with increased lung inflammation (Sundar et al. 2015). As future studies elucidate the interrelationship between circadian rhythm and COPD, new therapeutic targets and approaches will likely emerge.

2.3.2 Idiopathic Pulmonary Fibrosis (IPF)

IPF is a chronic, progressive, and irreversible idiopathic interstitial pneumonia of unknown origin. The mean survival is approximately 3–5 years from the time of diagnosis with a rate of progression that is highly variable and heterogeneous (Raghu et al. 2011; Bjoraker et al. 1998; Flaherty et al. 2002; Nicholson et al. 2000). IPF is an interstitial lung disease that is primarily associated with advanced age (Thannickal 2013). The disease is characterized by temporal heterogeneity of fibrosis characterized by clusters of actively proliferating fibroblasts/myofibroblasts, fibroblastic foci", and honeycomb structures (Raghu et al. 2011). TGF-β1 is a key regulator of myofibroblast differentiation and collagen deposition. Studies using animal models of pulmonary fibrosis, as well as in vitro studies using human epithelial cells and normal lung fibroblasts, revealed that TGF-β1 alters circadian rhythmicity and promotes Bmal expression while reducing Bmal1 acetylation (Dong et al. 2016a). Importantly, Bmal1 acetylation occurs in vivo and is regulated in a circadian manner. Bmal1 acetylation facilitates Cry1 interaction with the Clock–Bmal1 complex (Hirayama et al. 2007). The studies suggest that fibrogenesis occurs concomitant with significant changes in Bmal1, leading to dysfunctional clock regulation (Dong et al. 2016a). Furthermore, reduction of Bmal1 damped the effects of TGF-β1 on epithelial-mesenchymal transition and cell migration (Dong

et al. 2016a). The results support the role of the circadian clock in the expression of profibrotic genes involved in mesenchymal transition and invasion, relevant for lung cancer and pulmonary fibrosis.

2.3.3 Obstructive Sleep Apnea (OSA)

Ten percent of the U.S. population is affected by Obstructive Sleep Apnea (OSA), defined by repetitive episodes of obstruction in the upper airway during sleep, leading to chronic intermittent hypoxemia, sleep fragmentation, and chronic fatigue. Remarkably, OSA patients experience few or no problems with their breathing or airway patency while awake (Dempsey et al. 2010). Enlargement of soft-tissue structures both within and surrounding the airway contributes significantly to pharyngeal airway narrowing in most cases of OSA. Accumulation of even relatively small amounts of edematous fluid enlarges upper-airway soft-tissue structures in OSA patients and snorers, especially in the soft palate, which may be tugged caudally and constricted during apnea. Obesity also contributes indirectly to upper airway narrowing, and a broad range of candidate genes that might link genetic mechanisms of obesity with OSA are under investigation (Patel 2005; Young et al. 2002; Dempsey et al. 2010). Recent studies in mice revealed that obesity does not predispose mice to increased occurrence of central or obstructive apnea during sleep, but does lead to more pronounced circadian variability in respiration (Davis et al. 2013). Importantly, sleep-disordered breathing is recognized as a risk factor for the development of hypertension and other cardiovascular diseases. Sleep disruption per se increases sympathetic nerve activity and blood pressure (Morgan et al. 1996). Cardiovascular structure and function also change via neurohumoral activation, oxidative stress, and inflammation in OSA-induced cardiovascular morbidity and mortality (Dempsey et al. 2010). COPD and OSA have detrimental effects that are synergistic. Their comorbid association leads to compromised gas exchange (hypoxia and hypercapnia) and higher rates of morbidity and death (Khatri and Ioachimescu 2016). In animal models, OSA has been linked to metabolic syndrome, inflammation, hypertension, stroke, and cancer (Truong et al. 2016; Arias et al. 2006; Arble 2015, #6506). The prevalence of OSA is increasing, especially in the middle-aged population.

Sleep loss induces tissue-specific epigenetic and transcriptional alterations to circadian clock genes (Cedernaes et al. 2015). Therefore, it is expected that sleep disruption in patients with OSA leads to further changes in the circadian clock genes and their target genes. Recent population studies reveal that advancing age uniquely and robustly predicts OSA in females and reinforces the understanding that age-related changes in sex hormones play a role in the development and/or manifestation of sleep-disordered breathing (Cairns et al. 2016). There is a need for future studies to determine the circadian rhythms and sex differences in co-morbidity incidence in patients with OSA.

2.3.4 Infectious Disease and Vaccine Efficacy in the Elderly

It is possible that infectious agents exploit the circadian clock machinery. Circadian regulation has been shown to be altered in patients with pulmonary tuberculosis, as indicated by circadian deregulation of plasma hormones, including adrenocorticotropic hormone, which regulates the levels of cortisol; follicle-stimulating hormone; luteinizing hormone; hydrocortisone; testosterone; progesterone; and estradiol. The results of a study of 12 healthy subjects and 32 patients with infiltrative lung tuberculosis indicate that healthy subjects show fluctuations in the hormones that come to an acrophase in the morning hours, with stable time and amplitude structure. In contrast, tuberculosis patients exhibited impaired circadian cycling of the hormones, with acrophase in day and evening hours, reduced mean levels and amplitude in fluctuation, and non-coincidence in time (Karimdzhanov et al. 1993; Singh et al. 1991). Plasma melatonin levels are also lower in patients with pulmonary tuberculosis (Ozkan et al. 2012).

Recent work with influenza virus suggests that the virus directly exploits the deregulation of the clockwork (Edgar et al. 2016). Influenza virus infection is a public health problem that is generally more severe in individuals greater than 65 years of age. Respiratory infections can constitute major triggers of exacerbations of asthma and COPD through a robust host inflammatory response and an increase in bronchial hyper-responsiveness (Murray et al. 2004; Traves and Proud 2007). A recent study indicates that influenza virus infection promotes chronic CS-mediated inflammation and fibrosis (Sundar et al. 2015). Innate immune pathogen recognition mechanisms are under circadian control, as demonstrated by TLR9 expression and function (Silver et al. 2012). Conversely, viral and bacterial infection can alter the timing and amplitude of clock gene expression in the lungs. Viral respiratory infection mediated by influenza A causes molecular clock dysfunction in the lungs and increases mortality in Bmal1 knockout mice, suggesting a deficiency in the immune response to respiratory infection (Sundar et al. 2015). Mice with deficits in growth hormone releasing hormone signaling respond to influenza virus challenge with a progressive decrease in sleep and lower survival rates. The sleep response to influenza infection is mediated, in part, by regulation of hypothalamic sleep-related transcripts and enhanced corticosterone secretion (Alt et al. 2007).

Circadian clock regulation is critical for bronchial immune responses and the function of bronchial glucocorticoid receptors. The targeting of *Bmal1* in bronchiolar exocrine cells (Clara cells), leads to an exaggerated but apparently ineffective neutrophilic inflammatory response to bacterial infection, including to *Streptococcus pneumoniae* (Gibbs et al. 2014; Nouailles et al. 2014). At the molecular level, deficiency in Bmal 1 contributes to altered glucocorticoid receptor occupancy at the Cxcl5 locus and leads to enhanced Cxcl5 expression, implicated in tissue remodeling and polymorphonucleocyte-driven destructive inflammation in pulmonary tuberculosis (Gibbs et al. 2014; Nouailles et al. 2014).

A recent work revealed that Bmal1 selectively contributes to granulocyte circadian regulation in the endotoxemic lung (Haspel et al. 2014). Endotoxemia can also disrupt the circadian expression and periodicity of core molecular clock genes in murine lung, with some becoming arrhythmic and others showing a distorted but rhythmic pattern compared with baseline (Haspel et al. 2014). In consequence, endotoxins reprogram the circadian lung metabolome. The authors demonstrated in the lungs, a rhythmic accumulation of urate, a terminal product of purine metabolism, which was found to correlate with rhythmic infiltration of granulocytes producing myeloperoxidase, a catalytic enzyme responsible for the conversion of urate to allantoin.

Taking into account that light and daily rhythms are thought to have a strong influence on immune function (Roberts 2000) and that events affecting the immune system can alter the response to vaccination, it is plausible that vaccination administered in the morning or in the evening might influence the immune response to the vaccine. Few published human studies have addressed this question. Studies concerning flu shot efficacy do not show an association between vaccination time and subject response, similar to the results of studies with hepatitis B vaccine (Langlois et al. 1995; Karabay et al. 2008). However, it was demonstrated that the feelings of stress and loss of sleep become locked into a feed-forward circuit that ultimately diminishes the immune response against influenza virus after vaccination (Pedersen et al. 2009; Miller et al. 2004).

2.3.5 Lung Cancer

Dysfunction of the circadian clock is involved in tumorigenesis, and altered expression of some clock genes has been found in cancer patients and may be related to the process of tumorigenesis (Mazzoccoli et al. 2011).

Prolonged subjection to unstable work or lighting schedules, particularly in rotating–shift-workers, is associated with an increased risk of immune-related diseases, including several cancers. In 2007, The International Agency for Research on Cancer (IARC) concluded, “Shiftwork that involves circadian disruption is probably carcinogenic to humans” (Group 2A classification) (Straif et al. 2007). Lung cancer is one of the most deadly cancers, contributing to over a quarter of all cancer deaths in the United States. Lung cancer is considered an age-associated disease, whose progression is in part due to the accumulation of genomic instability and the age-related decline in system integrity and function (Levine et al. 2015). Association of clock genes with lung cancer was determined in 78 Brazilian patients with non-small cell lung cancer (NSCLC) (Couto et al. 2014). Those studies revealed that polymorphisms in the clock control gene nocturnin (*CCRN4L/NOC*) and *PER3* may represent a risk factor in the occurrence and development of NSCLC in Brazilian patients (Couto et al. 2014).

Chronic shift-lag can alter the circadian expression of clock genes *Per2* and *Bmal1*, and the cytolytic factors perforin and granzyme B, as well as the cytokine

IFN-γ. These alterations correlate with suppressed circadian expression of natural killer cell cytolytic activity and promotion of lung tumor growth (Logan et al. 2012). There is also evidence that a severe alteration in growth hormone-insulin-like growth factor axis function in patients with lung cancer is concomitant with loss of circadian rhythmicity of hormone secretion (Mazzoccoli et al. 2012).

Finally, it seems that adenocarcinoma of the lung functions as a potent endogenous circadian organizer in other tissues, rewiring the pathophysiological dimension of a distal tissue such as the liver. This was recently demonstrated by high-throughput transcriptomic and metabolomics studies, revealing a unique signature of transcripts and metabolites cycling exclusively in livers of tumor-bearing mice (Masri et al. 2016).

For this reason, it has been proposed that behavioral, hormonal, and/or light-based strategies to improve circadian organization may help patients suffering from advanced lung cancer to increase the quality of life, as a consequence of profound circadian disruption. (Grutsch et al. 2011). Future investigations will be important to strengthen current knowledge and shed light on the pathophysiology of circadian disruption on cancer risk in humans.

2.3.6 Asthma

The prevalence of asthma in the elderly affects more than 10% of patients of 60 years of age or older, while the estimated prevalence for COPD represents a 20–30% in patients older than 70 years of age (Hardie et al. 2005; Murtagh et al. 2005). Approximately 70% of adult asthma-related deaths occurred between 12:00 A.M and 6:00 A.M (Litinski et al. 2009).

Lung function varies in a circadian rhythm in healthy individuals. An episode of nocturnal asthma exacerbates the normal variation in lung function from daytime to night-time (Calhoun 2003). At night, a significant change in β-adrenergic receptor density and function occurs in patients with nocturnal asthma (Szefler et al. 1991). Decreased serum epinephrine levels, increased vagal nerve tone and cholinergic activity, esophageal reflux, and increased circulating eosinophils are known as contributors of the late-night/early-morning deterioration of lung function in asthma patients (Martin et al. 1990; Bates et al. 1994). The airflow obstruction correlates with an increase in inflammatory cells specifically associated with an increase in the number of airway $CD4^+$ lymphocytes and their capacity to generate IL-5 (Kelly et al. 2004).

Bonnet and colleagues identified the circadian rhythms of airway responsiveness to histamine and methacholine and correlation with airway tone in patients with mild asthma (Bonnet et al. 1991). Asthma patients also have disordered circadian rhythms of salivary melatonin and cortisol (Fei et al. 2004). Other factors, such as late-phase response to allergen exposure, sleep apnea, and lung volume changes during sleep, may also play a role in nocturnal asthma symptoms. Some early studies underscore the effects of sleep per se in worsening asthma and increased

airway resistance in asthmatic shift workers. Those studies observed that a decline in the peak expiratory flow rate was related to the change in sleep schedule, as opposed to the time of day (Clark and Hetzel 1977). Taking in account that the peripheral clocks of the lung and the immune system seems to be strongly interrelated, it is expected that the timing of an attack on the lung immune system by an allergen, virus or a therapeutic intervention might eventually affect the asthma symptoms, by altering the immune response. Future research is needed to elucidate the role of the circadian clock in asthma.

2.4 The Circadian Clock Regulates the Markers of Aging. Implications for the Pathophysiology of Age-Related Lung Disease

The effects of chronology on molecular rhythms in the human lung are not well understood. Early studies using rat tissue explants demonstrated that, whereas no significant change occurred in some tissues, 50% of explants from the lung and the retrochiasmatic area of the suprachiasmatic nucleus showed major circadian deregulation with age. Nevertheless, circadian rhythms were initiated in these arrhythmic tissues by forskolin stimulation, indicating that the aged tissues retained the capacity to oscillate (Yamazaki et al. 2002). Human studies revealed that the elderly population shows various circadian disturbances, including dampened amplitude of rhythmicity and decreased responsiveness to light (Mitteldorf 2013). On the other hand, changes in the expression of circadian clock genes alter biological aging. Evidence shows that disruption of the circadian system is associated with premature aging in mice, but the molecular basis of this phenomenon is still unclear (Grosbellet et al. 2015). In this section, we will explore the current understanding of the relationship between chronological aging, circadian clock, and biologic lung aging, taking into consideration that the lung is a vital organ constantly responding to insults.

The idea that circadian clock regulation can modulate the rate of aging is supported by earlier studies in which transplantation of pineal gland from young to old mice prolonged life span (Lesnikov and Pierpaoli 1994). Between 10 and 20% of the genes in any given cell are regulated by the circadian machinery, so it is expected that the hallmarks of aging can be regulated by circadian rhythms.

The term “hallmarks of aging” was first coined by Lopez-Otin et al. (2013) to describe cellular and molecular factors that contribute to the aging process. Those hallmarks are manifested during normal aging. Experimental aggravation accelerates aging; and conversely, its experimental amelioration can retard the aging process and increase life span (Lopez-Otin et al. 2013). The hallmarks include genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication (Lopez-Otin et al. 2013).

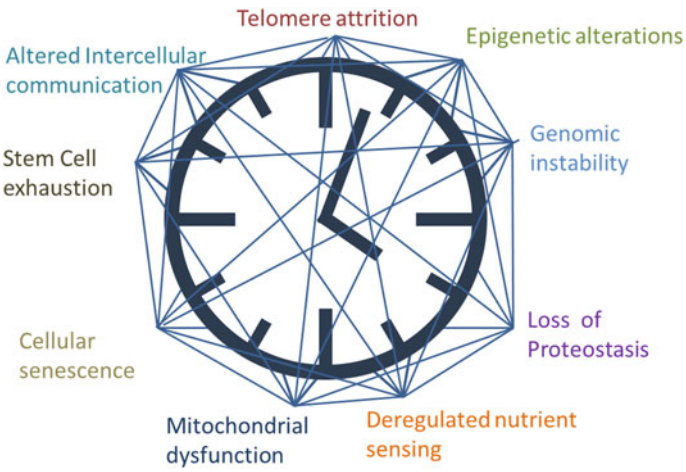

Fig. 2.2 Representation of the circadian clock regulation of biological aging

We now recognize that those hallmarks are tightly interconnected and act as mediators of the development and progression of age-related diseases. A major question remaining is how circadian systems regulate the hallmarks of biological aging, and how they influence "healthy aging" or age-related lung diseases (Fig. 2.2).

2.4.1 Telomere Attrition

Recent studies in humans and mice revealed that telomerase activity exhibits endogenous circadian rhythmicity. Specifically, those studies revealed that expression of telomerase reverse transcriptase (TERT) mRNA is under the control of CLOCK-BMAL1 heterodimers and that a loss of rhythmic telomerase activity leads to shortened telomere length. Furthermore, the authors described an increase in phosphorylation of histone H2A variant H2AX, a marker of DNA damage, in Clock-deficient mouse embryo fibroblasts (MEFs) after stimulation with staurosporine (Chen et al. 2014).

Several studies indicate that disease severity and smoking status in COPD, including perpetuation of lung inflammation, correlate directly with reduced telomere length in alveolar, endothelial, and smooth muscle cells, as well as in circulating lymphocytes (Amsellem et al. 2011; Tsuji et al. 2004; Albrecht et al. 2014). Concerning IPF, telomere attrition is a known driving factor in lung fibrosis. Mutations in telomerase genes have been found in 8–15% of familial and in 1–3% of sporadic cases of pulmonary fibrosis (Armanios 2013; Satoh 2016; Meiners et al. 2015). A negative slope in a plot of telomere length against age was found to be steeper in IPF patients than in controls, suggesting an accelerated rate of telomere

erosion in IPF patients. Furthermore, telomere length was found to be an independent predictor of survival in IPF (Dai and Gao 2016).

In asthma, telomere shortening was recently found to correlate with bronchial cell senescence. Those studies provide evidence that fibroblasts of adult asthmatic patients show significantly accelerated aging, which suggests a potential role of the continuous production of inflammatory mediators (Hadj Salem et al. 2015). With OSA, shorter telomere length was found in circulating leukocytes in patients compared to subjects without OSA (Barcelo et al. 2010). In patients with lung cancer, reduced telomere length is associated with poor prognosis (Frias et al. 2008). Specific studies are needed to understand the role of clock and telomere attrition in the development and progression of age-related lung diseases.

2.4.2 Genomic Instability

The clock gene machinery controls DNA-damage recognition and repair (Manzella et al. 2015; Kang et al. 2010). The circadian clock protein cryptochrome 2 interacts with TIMELESS, a primary regulator of the DNA replication machinery (Benna et al. 2010), and partner of the cell-cycle checkpoint protein Chk1 playing a major role in the DNA-damage checkpoint response (McFarlane et al. 2010). TIMELESS is critical for lung morphogenesis and correlates with poor survival of patients with lung cancer (Xiao et al. 2003; Yoshida et al. 2013).

Smoking is one of the most important environmental factors known to cause DNA damage and change the expression of circadian clock genes (Wistuba et al. 2002; Hwang et al. 2014). Nevertheless, alveolar epithelial cells and endothelial cells in patients with COPD and IPF have higher levels of DNA damage and damage response than those in asymptomatic smokers and nonsmokers (Aoshiba et al. 2012; Korfei et al. 2011; Kuwano et al. 1996), suggesting progression of DNA damage during disease progression. Dust mite-induced asthma causes oxidative damage and DNA double-strand breaks in the lungs (Chan et al. 2016). Thus, DNA damage can have a potential role in the pathogenesis of asthma (Chan et al. 2016). Unfortunately, little is known about the possible role of allergen-induced DNA damage and DNA repair as modulators of asthma-associated pathology.

2.4.3 Epigenetic Alterations

Whole-blood DNA methylation patterns change with chronological age, and multiple CpG sites with replicable associations with age have been identified (Florath et al. 2014). Epigenetic regulation is also regulated by the circadian clock, as indicated by recent observations that histone and DNA methylation are highly dynamic processes with more than 100 DNA methylation sites that oscillate in synchrony with the cell cycle (Brown et al. 2007), a cycle that is gated by the

circadian clock (Merrow and Roenneberg 2004; Nagoshi et al. 2004). The results of recent studies suggest that epigenetic age acceleration, based on DNA methylation age at 351 loci in 1820 subjects, is correlated with frailty, a clinically relevant aging-related phenotype, through pathways unrelated to cellular senescence as assessed by telomere length (Breitling et al. 2016). Several studies link epigenetic alterations with chronic lung disease. Using data on 2029 females from the Women's Health Initiative, a national survey that began in 1993 and enrolled postmenopausal women between the ages of 50 and 79 years (1998), one study identified DNA methylation age of blood as a predictor for future onset of lung cancer, and this association was even stronger in older individuals. The results indicated that epigenetic marks can be used as biomarkers for lung cancer susceptibility (Levine et al. 2015). Nevertheless, the value of the epigenetic clock in age-related lung diseases is currently unknown.

2.4.4 Loss of Proteostasis

The autophagy-lysosomal system and the ubiquitin-proteasomal system are regulators of protein quality control. Basal autophagy and other metabolic pathways are also rhythmically activated in a clock-dependent manner (Ma and Lin 2012). Recent studies indicate that proteolytic systems contribute to chronic lung diseases. Specifically, aberrant proteostasis contributes to COPD, severe emphysema, and pulmonary fibrosis (Bouchecareilh and Balch 2011; Sosulski 2015).

Autophagy has significant physiological significance for the lung, and alterations in autophagy have been implicated in the pathogenesis of various pathological lung conditions, including COPD and IPF (Sanchez 2016b). Autophagy has been shown to be reduced in IPF. Deficient autophagy induces acceleration of epithelial cell senescence and myofibroblast differentiation of lung fibroblasts and the progression of IPF (Patel et al. 2012; Araya et al. 2013; Ricci et al. 2013). The role of autophagy in the pathogenesis of COPD/emphysema appears to be complex and cell–type-specific. Mitophagy in alveolar epithelial cells contributes to the pathogenesis of COPD (Mizumura et al. 2014) and autophagy-deficient mice are protected from CS-associated ciliary dysfunction (Cloonan et al. 2014). Nevertheless, recent studies indicate that impairment of CS-induced autophagy accelerates lung aging, COPD-emphysema exacerbations, and pathogenesis (Vij et al. 2016).

Rhythmic autophagic induction may be essential for temporal remodeling of proteomes and organelles. Periodic removal of mitochondria and other organelles may facilitate the adjustment of the bioenergetic properties throughout different circadian phases (Ma et al. 2012). Circadian regulation of several transcriptional regulators of autophagy genes is mediated by transcription factor CEBPB, linking the circadian clock to autophagy and maintenance of nutrient homeostasis in mice (Ma et al. 2011). In addition to the role of transcription factors, studies in zebrafish indicate that the circadian clock directly regulates autophagy genes, specifically Nr1d1 and Per1b, which are critical for maintaining autophagic rhythms in

zebrafish (Huang et al. 2016). Furthermore, pathways involved in autophagic regulation, such as the mTOR and AMPK pathways, appear to undergo circadian regulation; however, their roles in driving rhythmic autophagy activation in lung tissue remains to be explored. Interestingly, studies in MEFs revealed that treatment with lysosomal inhibitors significantly enriches the protein Bmal1 to a degree comparable to that achieved with MG132 treatment, indicating that autophagy and the proteasome plays a major role in Bmal1 degradation (Jeong et al. 2015).

Another mechanism of proteostasis is the proteasome, which regulates Bmal1/Clock function but also exhibits circadian rhythmicity in expression level and activity (Stratmann et al. 2012; Desvergne et al. 2016). The healthy aging of the lung does not involve impairment of proteasome function (Caniard et al. 2015). By contrast, COPD and direct exposure to cigarette smoke alter pulmonary proteasome expression and activity which inversely correlate with lung function (van Rijt et al. 2012).

The rhythms match the circadian oscillations in oxidative protein damage (Desvergne et al. 2014). Using synchronized human embryonic kidney (HEK) 293 cells and primary dermal fibroblasts, it was shown that the levels of carbonylated protein and proteasome activity vary rhythmically following a 24-h period, in part due to the circadian expression of a nuclear factor, erythroid 2 like 2 (NRF2) and the proteasome activator PA28α/β. Interestingly, no circadian modulation of proteasome activity or carbonylated protein level was observed in senescent fibroblasts compared to control fibroblasts (Desvergne et al. 2016). Therefore, it is plausible that age-associated alterations in the circadian systems may contribute to altered proteasome activity and accumulation of oxidized proteins in age-related lung diseases.

Stress signals lead to selective activation of downstream signaling cascades, including activation of PERK, a type I membrane protein located in the endoplasmic reticulum, which phosphorylates the translation initiation factor eIF2α, leading to attenuation of global protein synthesis (Harding et al. 2000). Inhibition of eIF2α allows selective expression of ATF4, a regulator of genes involved in protein folding, antioxidant responses, autophagy, amino acid metabolism, and apoptosis (Harding et al. 2003). Active PERK phosphorylates and activates NRF2, a master regulator that promotes cell survival by counteracting oxidative stress and modulating redox signaling (Niture et al. 2010; Hybertson et al. 2011). Altered NRF2 expression has been associated with the pathogenesis of chronic lung diseases, such as asthma, COPD, and IPF, contributing to excessive oxidative stress in the lung (Cho and Kleeberger 2007; Cho et al. 2006). Importantly, NRF2 is known to be regulated in a circadian manner (Pekovic-Vaughan et al. 2014). Therefore, circadian control of the NRF2/glutathione pathway plays a significant role in combating oxidative/fibrotic lung damage, which might prompt new chronotherapeutic strategies for the treatment of human lung diseases, including IPF (Pekovic-Vaughan et al. 2014; Malhotra et al. 2011; Kumar et al. 2011).

2.4.5 Deregulated Nutrient Sensing

NAD+ is a promising candidate to be an integrator of circadian-rhythm and nutrient-sensing pathways. Several studies have demonstrated that the circadian clock regulates the synthesis of the essential metabolic cofactor nicotinamide adenine dinucleotide (NAD+), which plays a central role in redox reactions and is an important cofactor of the class III histones and protein deacetylases (sirtuin family of NAD+-dependent deacetylases). CLOCK:BMAL1 directly regulates nicotinamide phosphoribosyltransferase (NAMPT) transcripts and NAD+ levels (Peek et al. 2012; Ramsey et al. 2009; Nakahata et al. 2009). In circadian mutant mice, NAD+ supplementation restores protein deacetylation and enhances oxygen consumption (Peek et al. 2013).

Class III deacetylases participate in both protein deacetylation and ADP-ribosylation and constitute a rapid means of upregulating mitochondrial energy production during nutrient deprivation. SIRT1 and SIRT3 are members of the sirtuin family that regulates a range of metabolic processes, senescence, and life span (Saunders and Verdin 2007; Donmez and Guarente 2010; Haigis and Sinclair 2010; Peek et al. 2012). Sirt1 and Sirt3 have been implicated in the pathogenesis of age-related lung diseases, such as COPD and IPF. Sirt1 is decreased in macrophages and lungs of smokers and COPD patients, leading to an increase in NF-κB–dependent proinflammatory mediators in lung and markers of oxidative and nitrosative stress (Rajendrasozhan et al. 2008). Others showed that the progressive decline in Sirt1 is accompanied by increasing levels of IL-8 and MMP9 with increasing disease severity, whereas induction of SIRT1 protects against COPD/emphysema in animal models and contributes to correcting the imbalance in the TIMP-1/MMP-9 ratio (Yao et al. 2013). Bmal1 is acetylated and degraded in the lungs of mice exposed to CS and in patients with COPD, compared with lungs of nonsmoking controls, linking it mechanistically to CS-induced reduction in SIRT1 (Hwang et al. 2014).

Circadian control of the activity of SIRT3 generates rhythms in the acetylation and activity of oxidative enzymes and respiration in isolated mitochondria. In liver of *Bmal1*$^{-/-}$ mice, acetylation is increased at specific lysine residues of the antioxidants MnSOD (Lys^{122}) (Tao et al. 2010) and IDH2 (Lys^{413}) (Yu et al. 2012), known targets of Sirt3. Thus, it was demonstrated that Sirt3 activity is under the control of the circadian clock (Peek et al. 2013). Aging and TGF-β treatment lead to Sirt3 deficiency, acetylation of MnSOD and IDH2, and promotion of pulmonary fibrosis (Sosulski et al. 2016).

2.4.6 Mitochondrial Dysfunction and Oxidative Stress

In mitochondria, both metabolic and cellular defense mechanisms are carefully regulated. Mitochondrial instability has been reported in lung cancer, suggesting

that mitochondrial dysfunction contributes to the progression of lung cancer and multidrug resistance (Mambo et al. 2005; Kamp et al. 2011; Lee et al. 2015; Ma et al. 2015).

Abnormal clock function with aging might influence mitochondrial function. Mutations in mtDNA and oxidative DNA damage have been observed to accumulate in the lung and other tissues during human aging. Several studies suggest that the increase in mtDNA content of aging tissues may be effected through a feedback mechanism to compensate for the functional decline of mitochondria in human aging, and that smoking may modulate the mechanism (Lee et al. 1998, 1999). It is also possible that aging is concomitant with deficient autophagic degradation of mitochondria, a mechanism that can itself be circadian regulated, leading to accumulation of dysfunctional mitochondria (Sosulski et al. 2015; Jacobi et al. 2015). Similarly, exposure to CS impairs mitophagy (Ahmad et al. 2015). There are few studies connecting asthma with mitochondrial dysfunction; however, a recent study demonstrates a significant reduction in mitochondrial glucocorticoid and estrogen receptors in human bronchial epithelial cells from fatal asthma cases (Simoes et al. 2012).

During aging, autophagic clearance of mitochondria declines and dysfunctional mitochondria provoke chronic oxidative stress, which disturbs the cellular redox balance (Salminen et al. 2012b). Reduced mitochondrial oxidative capacity and abnormal coupling are also evident as a consequence of perinatal nicotine exposure, but the mechanisms need to be elucidated (Cannon et al. 2016). Exposure to airborne particulate matter, as well as to profibrotic factor TGF-β, leads to a decrease in mitochondrial respiratory function (Delgado-Buenrostro et al. 2013; Sosulski et al. 2015). It is possible that the environmental induced mitochondrial dysfunction can be associated to circadian changes. According to the circadian gene expression database (http://expression.gnf.org/circadian), several respiratory chain components, including subunits of complex I, appear to display a circadian expression pattern in mice, and a number of others that remain to be analyzed contain E-box motifs for clock transcription factor complexes in their promoters (Hogenesch et al. 1998). Deficiency in Sirt3 also alters antioxidant response and mitochondrial dynamics and function. Therefore, changes in the circadian regulation of Sirt3 might have significant consequences for mitochondrial function and response to injury.

It is also evident that mitochondrial proteins, such as prohibitins, can modulate the internal circadian clock. Prohibitins are versatile proteins located in the inner mitochondrial membrane and involved in mitochondrial function and morphology. Prohibitin 2 (PHB2) is a modulator of period length (Kategaya et al. 2012). Downregulation of PHB2 increases circadian-driven transcription, thus revealing that PHB2 acts as an inhibitor in the molecular clock. Even if no significant differences are found in PBH2 expression in COPD, prohibitin 1 (PHB1) was previously found to be significantly downregulated in bronchial epithelial cells of patients with COPD and likely contributes to oxidative stress in the COPD lung (Soulitzis et al. 2012).

Several defects in mitochondrial dynamics, including mitophagy, have been reported in chronic lung diseases (Sosulski et al. 2015). Reduced expression of PINK1 is observed in the lungs of aging mice and IPF patients and is associated with pulmonary fibrosis (Sosulski et al. 2015; Bueno et al. 2014). Changes in mitochondrial dynamics may also be relevant to the pathogenesis of COPD (Hara et al. 2013; Hoffmann et al. 2013; Aravamudan et al. 2014). It has been shown that mitochondria of primary bronchial epithelial cells from COPD patients show elongation and fragmentation, swelling, and depletion of cristae (Hoffmann et al. 2013). Mitochondrial dysfunction is also linked to inflammatory responses (Escames et al. 2013; Lopez-Armada et al. 2013). Thus, strategies aimed at controlling excessive oxidative stress within mitochondria might have a therapeutic purpose against inflammation (Lopez-Armada et al. 2013, Yue and Yao 2016).

Mitochondrial dysfunction in muscle cells may contribute to the loss of muscle strength, leading to a decline in physical function, which is a systemic manifestation of COPD (Lloreta et al. 1996; Mayer et al. 2013). Currently, mitochondria-targeted interventions are being developed in studies to elucidate the role of mitochondrial metabolism and recycling in lung aging, COPD, and pulmonary fibrosis.

2.4.7 Cellular Senescence

Cellular senescence and the inflammatory profile of senescent cells play significant roles in the pathogenesis of chronic lung diseases, including COPD and lung fibrosis (Tsuji et al. 2010; Aoshiba and Nagai 2009; Bartling 2013; Chilosi et al. 2013). Senescent cells recruit the immune system to facilitate their removal from tissues. Nevertheless, during ageing, their senescence-associated secretory phenotype (SASP) drives major pro-inflammatory consequences (Ovadya and Krizhanovsky 2014). On one hand, senescence decreases the ability of cells to transmit circadian signals to their clocks. Conversely, the introduction of telomerase completely prevents this reduction of clock gene expression associated with senescence (Kunieda et al. 2006). On the other hand, deficiency in circadian clock regulation can result in senescence. In fact, deficiency of Bmal1 results in the development of premature aging in mice, with an increase in the number of senescent cells in different tissues (lungs, liver, and spleen). However, it appears that Bmal1 doesn't play a significant role in replicative senescence, as demonstrated by similar rates of proliferation and senescence in primary fibroblasts isolated from wild-type and *Bmal1*$^{-/-}$ mice (Khapre et al. 2011).

2.4.8 Stem Cell Exhaustion

The influence of circadian rhythm on the regulation of stem cells has recently begun to be evaluated. Clocks have been suggested to underlie heterogeneity in stem cell

populations to optimize cycles of cell division during wound healing (Brown 2014). A link is also emerging between the circadian clock and metabolic transcriptional regulation by epigenetic mechanisms, implying a role of this interaction in stem-cell homeostasis (Avitabile et al. 2016). Nevertheless, little is known about the role of circadian rhythm in human mesenchymal bone marrow stem cell (BMSC) properties. Rev-erbα and the Wnt/β-catenin signaling pathway are known to play important roles in BMSC aging, and it has been suggested that Rev-erbα may promote BMSC aging and function as a negative regulator during late-stage osteogenesis (He et al. 2015). Recent data confirm that circadian rhythms also play a role in the regulation of mesenchymal stem cell differentiation and division, key factors in maintaining mesenchymal stem cell properties (Boucher et al. 2016). Stem cell-based therapy is being proposed for the treatment of chronic lung diseases (Gore et al. 2016; Lin et al. 2015; Zhang et al. 2014; Pierro and Thebaud 2010). In consequence, there is a need to determine the role of the circadian clock in the maintenance of lung progenitor cells, specifically, the role the circadian clock regulators play in stem cell maintenance to preserve lung integrity during aging and tissue repair. The identification of factors that potentiate the regenerative capacity of stem cells is crucial for the development of next-generation therapeutics for chronic lung diseases.

2.4.9 Intercellular Communication

A prominent aging-associated alteration in intercellular communication is the proinflammatory phenotype that accompanies aging in mammals (Salminen et al. 2012a, b). Circadian rhythms mediated by light/dark cycles influence the immune functions of natural killer cell activity, lymphocyte and monocyte proliferation and their secretome (Boivin et al. 2003). Eight percent of all mRNA transcripts in macrophages show oscillation of expression over a 24-h period. Bronchiolar epithelium is part of the cell-specific timing mechanism that regulates overall homeostasis of immunological responses (Gibbs et al. 2014). Conversely, cytokines in the lung can also reprogram the circadian rhythm (Haspel et al. 2014; Dong et al. 2016b).

Intercellular communications are also highlighted by exosome secretion. Exosomes exhibit pleiotropic biological functions, including immune regulatory functions, antigen presentation, intracellular communication, and intercellular transfer of RNA and proteins. Exosomal protein and microRNA (miR) content reflects the physiological condition of the cells of origin, as presented in an interesting study with lung adenocarcinoma cells (Choi et al. 2014). Importantly, sleep fragmentation induces alterations in biosynthesis and cargo of plasma exosomes that affect tumor cell properties (Khalyfa et al. 2016). Exosomes can also enhance the transcriptional activity and abundance of β-catenin, a primary regulator of epithelial-mesenchymal transition (Choi et al. 2014). CS can modify the extra-vesicular components from bronchial epithelial cells, such as exosomes carry

miR-210 as paracrine mediators of myofibroblast differentiation (Fujita et al. 2015). To our knowledge, no studies have been done concerning the circadian regulation of exosome secretion and content in chronic lung diseases. Conversely, it appears that exosomes can regulate circadian gene expression, as shown in studies in *Neurospora* (Guo et al. 2009). It will be important to determine if exosomes have a circadian regulatory potential in the lung.

Intercellular communication can also lead to inter-tissue communication. This is highlighted by a recent study revealing that adenocarcinoma lung functions as a circadian organizer that rewires a distal tissue, such as the liver, through an altered proinflammatory response via the STAT3-Socs3 pathway. Future studies will need to address how aging regulates inter-tissue communication (Cairns and Mak 2016; Masri et al. 2016).

2.5 Chrono-Therapy for Age-Related Lung Diseases

Chrono-pharmacology, or circadian regulation of drug function, as well as chrono-tolerance and chrono- nutrition, are likely to become important research fields in chronobiological studies (Mitteldorf 2013; Tahara and Shibata 2014).

Melatonin. A crucial observation was the decline of the nocturnal melatonin peak in elderly persons and the capacity of melatonin to prolong life span. Different subtypes of melatonin receptors may address the issue of the various physiological actions of melatonin reported in individual tissues within the same species or similar tissues in different species. Beneficial effects of melatonin in attenuating aging-related deterioration have been demonstrated in several publications (Armstrong and Redman 1991; Jenwitheesuk et al. 2014). In fact, melatonin modulates the inflammatory and apoptosis status of the aging lungs, exerting a protective effect on age-induced damage (Puig et al. 2016). One study, aiming to determine whether melatonin administration can prevent the hyper-oxidative state that occurs in lung mitochondria with age, concluded that melatonin protects lung mitochondria from aging with similar benefits in male and female mice (Acuna-Castroviejo et al. 2012). Melatonin also reduces lung injury in bleomycin models of pulmonary fibrosis regarding mortality rate, the degree of inflammation, and fibrosis (Karimfar et al. 2015; Zhao et al. 2014; Yildirim et al. 2006; Genovese et al. 2005). Melatonin was found to be significantly reduced during the exacerbation period in patients with COPD (Gumral et al. 2009). In contrast, a daily dose of melatonin has been shown to protect lungs from histopathological changes in rabbits exposed to smoke (Unlu et al. 2006). A 4-week study revealed that melatonin could improve sleep in patients with asthma; however, long-term studies are needed to resolve conflicting data (Campos et al. 2004; Luo et al. 2004). Concerning cancer cells, melatonin has an inhibitory effect on the reduction in mitochondrial membrane potential that occurs upon doxorubicin treatment and the development of premature senescence at the cellular level and protects adenocarcinomic human alveolar basal epithelial cells (A549 cells), from

doxorubicin-induced senescence (Song et al. 2012). Melatonin orally administered to reset the circadian clock reduces the toxicity of various chemotherapeutic agents (Lewy et al. 1999; Lewy and Sack 1997; Lissoni et al. 1994; Lissoni 2000). Melatonin also enhanced the antitumor activity of berberine through activation of caspase/cyto C and inhibition of AP-2β/hTERT, NF-κB/COX-2, and Akt/ERK signaling pathways (Lu et al. 2016). Other positive results in animal studies suggest that melatonin prevents the increase in glucose levels that usually follows intermittent exposure to hypoxia seen in OSA (Kaminski et al. 2015). Exogenously administered melatonin can also protect lungs from reperfusion injury after prolonged ischemia in transplant models (Inci et al. 2002).

Chrono-toxicity has been evaluated for some therapies, particularly cancer therapies. One early study demonstrated that cisplatin-based chronotherapy has the advantage of relieving side effects of the chemotherapy in patients with advanced NSCLC and that the metabolism of cisplatin is circadian regulated (Li et al. 2015). Nevertheless, little is known about chrono- pharmacology and chrono- toxicity of most drugs currently used to treat chronic lung conditions.

Small molecules in chronotherapy. Approximately 200,000 compounds have already been screened and characterized as circadian regulators acting as modifiers of period length, phase delay, phase advance, phase attenuation, and amplitude (Tahara and Shibata 2014). Through chemical-screening approaches, a number of compounds that affect circadian rhythms have been discovered, including those presented in Table 2.1, such as casein kinase I inhibitors and synthetic ligands for the nuclear receptors REV-ERB and ROR (Sprouse et al. 2010; Badura et al. 2007; Gibbs et al. 2012; Cho et al. 2012; Solt et al. 2012). Some of those compounds had been proposed previously as therapeutic alternatives for diseases such as lung cancer (Hung et al. 2013; Tang et al. 2016; Parajuli et al. 1999; Schwandt et al. 2012; Ferguson et al. 2015; Hayashi et al. 2006) and exacerbated inflammation in models of acute lung inflammation and COPD (Ratcliffe and Dougall 2012; Brando

Table 2.1 Summary of small molecules as circadian regulators

Circadian effect	Molecules
Period-lengthening activity	Casein kinase 1 inhibitor (Sprouse et al. 2010; Badura et al. 2007); MAP kinase p38 inhibitor (Dusik et al. 2014; Pizzio et al. 2003; Hayashi et al. 2003); JNK inhibitor (Pizzio et al. 2003); PP2A inhibitor (Yang et al. 2004)
Period-shortening activity	DNA topoisomerase II inhibitor, PKC agonist, CDK inhibitor, and GSK3β inhibitor (Hirota et al. 2008)
Attenuation of phase shifts	Kinase inhibitors, including U0126 (ERK) (Coogan and Piggins 2003), KN-62 (CaMKII) (Golombek and Ralph 1994), KT5823 (PKG), and SB431542 (ALK) (Kon et al. 2008)
Phase delay and amplitude enhancement	Inducer of cellular cAMP, phosphodiesterase inhibitor (rolipram), and secondary inducer of cAMP (Chen et al. 2012; Hirota et al. 2012)
Amplitude reduction	Agonists of Rev-erbα or Rev-erbβ (Gibbs et al. 2012; Cho et al. 2012; Solt et al. 2012)

Lima et al. 2011; Arndt et al. 2005). It will be critical in the near future to determine whether strategies known to facilitate synchronization of the circadian clocks in younger individuals can do so in older individuals, and whether doing so allows patients with chronic lung disease to experience improved quality of life and/or live longer. Thus, it will be beneficial to determine whether the use of the small molecules capable of manipulating the clock leads to beneficial effects in chronic lung diseases.

2.6 Future Directions

The potential for novel chrono-pharmacological approaches for the treatment of lung disease needs to be determined in the near future in agreement with recent lines of evidence indicating that aging proceeds under control of a master clock or several redundant clocks (Mitteldorf 2016). It is plausible that resetting the clocks with biochemical interventions might regulate the hallmarks of aging and make an old lung behave like a young lung. Developing strategies to prevent circadian disruption effectively with aging to prevent the onset of age-related lung diseases and to provide effective strategies to diminish circadian disruption among patients with chronic lung diseases are worthwhile and accomplishable goals.

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Chapter 3
Circadian Regulation of Bone

Sifat Maria and Paula A. Witt-Enderby

Abstract The demands of modern society are posing serious effects on our bone health. People are working longer hours, working through the night, getting less sleep, and eating at irregular hours. This is causing more stress and less time to spend outdoors. All of these factors are contributing to circadian disruption "in general" but more importantly to circadian disruption of bone rhythms. Bone metabolism displays circadian variation that is coincident with clock rhythms in bone, with the light/dark cycle and with circulating melatonin levels. Light exposure at night, shift work, and poor quality sleep can lead to weakened bones attributed, in part, to altered clock rhythms in bone and to changes in circulating melatonin and cortisol rhythms in the body. The intent of this review is not to describe bone metabolism "in general" and then to discuss the effect of melatonin in these processes. There are many reviews on this subject matter described throughout the chapter. Rather, the focus of this chapter is to describe clock gene expression and function in bone and how their rhythms impact on osteoblast and osteoclast activity and differentiation and on bone metabolism; and then discuss variables that lead to circadian disruption of bone rhythms and describe ways to maintain healthy bone in a society that continually promotes circadian disruption.

Keywords Bone rhythms · Melatonin · Osteoclasts · Osteoblasts · Shift work Circadian disruption

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3.1 Introduction

Up to one-third of the workforce in the United States engages in flexible work schedules that include weekends and nontraditional hours (early morning, evenings etc.) and one-fifth of the population works the night shift. According to the US Department of Labor in 2004, of Americans surveyed from 1985 to 2004, 15% of workers (full-time wage and salary workers) worked a shift other than a daytime schedule—half of those surveyed stated that this was due to the nature of the job itself and not personal preference. Similar trends were observed in Canada where approximately one-third of employed individuals work alternative shifts and, overall, it was also determined that in any urban society, an estimated one-fifth of people work alternative shifts (Navara and Nelson 2007).

As we become a shiftless society, more health problems will arise that are associated with circadian disruption including bone disease (Maria and Witt-Enderby 2014). In recent years, the effect of circadian disruption on bone has been getting more attention and this is following the publication of a few key studies demonstrating that shift work produces ill effects on bone. In one study examining the effect of shift work in nurses, Feskanich et al. (2009) determined that 20 years or more of shift work was associated with an increased risk of hip and wrist fractures compared to women who never worked night shifts (Feskanich et al. 2009). In another study examining the effect of rotating shifts on bone mineral density (BMD) and osteopenia in 70 postmenopausal nurses from the Naval Hospital in Concepcion, Chile, those who worked rotating shifts had lower BMD in lumbar spine and femoral neck bones; T-scores in 25.6% of rotating shift workers showed osteoporosis in the lumbar spine while no osteoporosis was observed in the day-time shift workers; and a higher prevalence (10.7% increase) of osteopenia was observed for rotating shift workers (Quevedo and Zuniga 2010). Another study examined the effect of shift work, defined as evening time, nighttime, regular and irregular shift work, on BMD in 3005 Korean subjects both male and female (18–50 years old) and demonstrated a lower BMD in lumbar spine and total femur and higher rates of osteopenia compared to those who worked the day shift (Kim et al. 2013). Because shift work impacts strongly on sleep patterns, sleep, like circadian disruption, may impact on overall bone health. This is supported in a study examining the association between sleep quality on BMD in pre- and post-menopausal women. In this study by Wang et al. (2015), delayed bedtime (after midnight), excessive daytime sleep (>3 h) and frequent daytime napping (>1 h) was associated with decreased BMD in postmenopausal women but not in pre-menopausal women (Wang et al. 2015).

Besides shift work, circadian disruption could be caused by business travel (specifically transmeridian travel), social jet lag, light exposure at night (LAN), sleep patterns, menopausal status and aging. For those with late chronotypes, the demand of an early work schedule during the week can lead to many hours of sleep debt when compared to the weekend. Social jet lag due to non-work related activities occurs when differences arise between one's circadian and social clock. It

is estimated that 70% of the population in the United States experiences social jet lag (Bonmati-Carrion et al. 2014). The impact of chronic sleep debt caused by business travel, social activities and work schedules on bone health are not clear and further investigation is warranted.

3.2 Circadian Rhythms and Bone

In humans, it has long been known that bone metabolism displays circadian variation (Schlemmer et al. 1992; Oliveri et al. 2008; Uebelhart et al. 1991; Greenspan et al. 1997; Eastell et al. 1992; Aoshima et al. 1998; Bollen et al. 1995; Heshmati et al. 1998; Nielsen et al. 1991). The intensity of these peaks increase with menopause (Uebelhart et al. 1991) and osteoporosis (Eastell et al. 1992) in women; and the peaks observed in males is about half of that to what is observed in women (Greenspan et al. 1997). All of these data suggest that lifestyle, gender, age, hormonal status, and degree of bone disease can influence the daily rhythms of bone metabolism. Perturbations in these rhythms could negatively affect bone and increase one's risk of bone disease as is seen in children with a genetic condition called Smith-Magenis syndrome—a condition that is associated with disrupted sleep rhythms, bone abnormalities and altered clock (PER2) rhythms (Novakova et al. 2012; Potocki et al. 2000).

3.2.1 Circadian Regulation of Clock Genes Expressed in Bone

The potential mechanisms driving these circadian rhythms in bone are through clock proteins, BMAL, CLOCK, CRY and PER located within bone itself. All of these proteins have been detected in calvarial or cancellous bone (Zvonic et al. 2007; Kondo and Togari 2015; Min et al. 2016; Witt-Enderby et al. 2012). In fact, in mouse calvarial bone, more than 26% of the genes present on the Mouse Genome 430A 2.0 Array (Affymetrix) displayed an oscillatory pattern (Zvonic et al. 2007) suggesting that clock proteins may be involved in the regulation of bone metabolism. For the most part, all of the studies to date demonstrate that the clock proteins expressed in bone display circadian variation throughout the light/dark cycle and the timing of their peaks and troughs are fairly consistent between studies described in Fig. 3.1. For Per2, its mRNA levels peak during the light/dark transition (ZT12) and trough during the dark/light transition in both calvarial and cancellous bone (Witt-Enderby et al. 2012; Kondo and Togari 2015; Zvonic et al. 2007). In two of the studies, Bmal was consistently anti-phase to Per2 (Zvonic et al. 2007; Kondo and Togari 2015) and its levels peaked at ZT0 (right at lights on) and troughed at ZT12 (right at lights off). Additional clock genes were analyzed in only one study

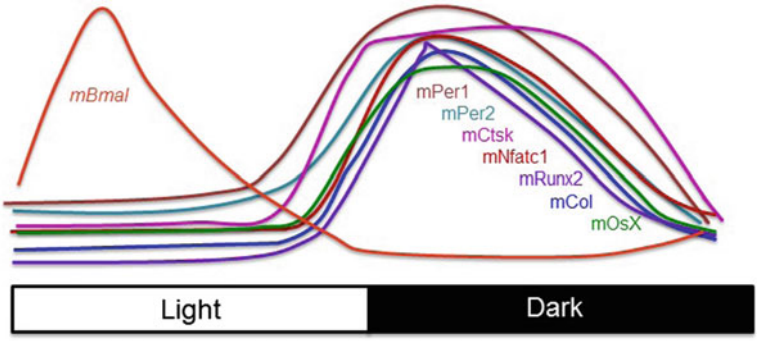

Fig. 3.1 Circadian rhythms of osteogenic genes. As depicted, clock genes, Period (Per) and brain and muscle ARNT-like (Bmal) genes display rhythms where Bmal is out of phase with Per. Per is coincident with the oseogenic genes, type 1 collagen (Col), runt-related transcription factor (Runx2), bone morphogenetic proteins 2 and 6, Bmp2, Bmp6, bone gamma-carboxy glutamate protein/osteoclcin, Bglap and the bone-resorbing genes, cathepsin K (Ctsk) and nuclear factor of activated T-cells cytoplasmic 1 (Nfatc1), which also display circadian variation throughout the 24 h cycle. Except for Bmal, most of these genes peak right before or at the onset of darkness (Zvonic et al. 2007; Kondo and Togari 2015; Witt-Enderby et al. 2012; Min et al. 2016)

(Zvonic et al. 2007) showing that Per1, 3, like Per2, had a zenith between ZT 8–12 (end of light cycle) and a nadir at ZT0; Cry 2 had a zenith at ZT 8–12 and a trough at ZT0; Cry 1 peaked at ZT20 and troughed at ZT4; and Clock did not display a consistent circadian profile.

3.2.2 Circadian Regulation of Osteoblast- and Osteoclast-Specific Genes

The impact of clock genes on bone was first discovered by Fu et al. (2005) and then by Maronde et al. (2010) who discovered that mice lacking Per or Cry in their osteoblasts displayed increases in bone mass. This was followed by another study by Samsa et al. (2016) demonstrating that a Bmal1 deficiency in mice reduced osteoblast activity and bone mass. All of these studies suggest that Per and Cry are important negative regulators of bone and Bmal is a positive regulator of bone. How clock protein circadian rhythms impact on osteogenesis is not clear but both osteoblastic (type 1 collagen, Col1, runt-related transcription factor, Runx2, osterix, Osx, bone morphogenetic proteins 2 and 6, Bmp2, Bmp6, bone gamma-carboxy glutamate protein/osteocalcin, Bglap) and osteoclastic (cathepsin K, Ctsk and nuclear factor of activated T-cells cytoplasmic 1, Nfatc1) genes display circadian rhythms where their levels peak right at the light/dark transition and then eventually decline by the end of the dark cycle (Zvonic et al. 2007; Kondo and Togari 2015; Witt-Enderby et al. 2012). Their rhythms are similar in timing with the clock protein shown in Fig. 3.1.

3.2.3 Mechanisms Underlying Clock-Mediated Regulation of Bone

The mechanisms underlying the relationship between clock proteins and osteoblast and osteoclast differentiation and activity are not clear but some are emerging described in Fig. 3.2. For example, Bmal, using Chromatin immunoprecipitation (ChIP) assays, has been shown to bind to two E-boxes (CACGTG) on osteoclast genes, *mCtsk* and *Nfatc1,* which explains why the period and phase of oscillation for mCtsk and mNfatc1 were similar to that of mPer1 and mPer2 (Kondo and Togari 2015). This implies clock genes can regulate osteoclast-related genes and impart their patterns of circadian variation to synchronize bone metabolism rhythms with the endogenous circadian clock. Clock-mediated regulation of osteoblasts has also been described, which can be further modulated by the light/dark cycle, melatonin, estrogen, and cortisol. This complicated dynamic between these variables is described in Fig. 3.2. Briefly, Clock:Bmal heterodimers in bone bind to an E-box located on Per and Cry driving their expression. Per:Cry heterodimers then attenuate their expression through a negative feedback loop. Besides binding to Cry to limit their expression, Per2 may be modulating bone by working through p21/cyclin D1/cyclin D1 kinase (CDK1) to inhibit the cell cycle. For example, Per2 can control cell division through its actions on cyclin D1 (Gu et al. 2012) where a loss of Per2 would increase G1/S and increase bone proliferation. This may explain, in part, why Per2-deficient mice display increases in both bone mass and volume (Fu et al. 2005; Maronde et al. 2010). Bmal, like Per2, has been shown to modulate bone independent of its role in mediating Per2/Cry expression by forming heterodimers with Clock. Overexpression of Bmal in mouse (MC3T3) cells increased osteoblast differentiation through increases in Bmp-2-mediated osteocalcin, Runx2, and inhibitor of DNA binding (Id1) mRNA expression (Min et al. 2016). This mechanism explains, in part, why Bmal-deficient mice displayed low bone mass and decreases in osteoblast function (Samsa et al. 2016).

3.3 Humoral Effects of Bone Rhythms: Melatonin, Estrogen and Cortisol

3.3.1 Melatonin Effects on Bone and Bone Rhythms

Melatonin production commences between ages 1 and 3 years and then becomes circadian in nature (Waldhauser et al. 1984) where it serves as the marker for the 24-h periodicity of the endogenous circadian system in light-sensitive sighted humans (Lockley 2007). The release of melatonin is rhythmic where levels are lowest during the biological day and highest during the biological night (Kennaway et al. 2002; Zawilska et al. 2009). These melatonin rhythms persist throughout adolescence and adulthood but then begin to decline with age and, in women,

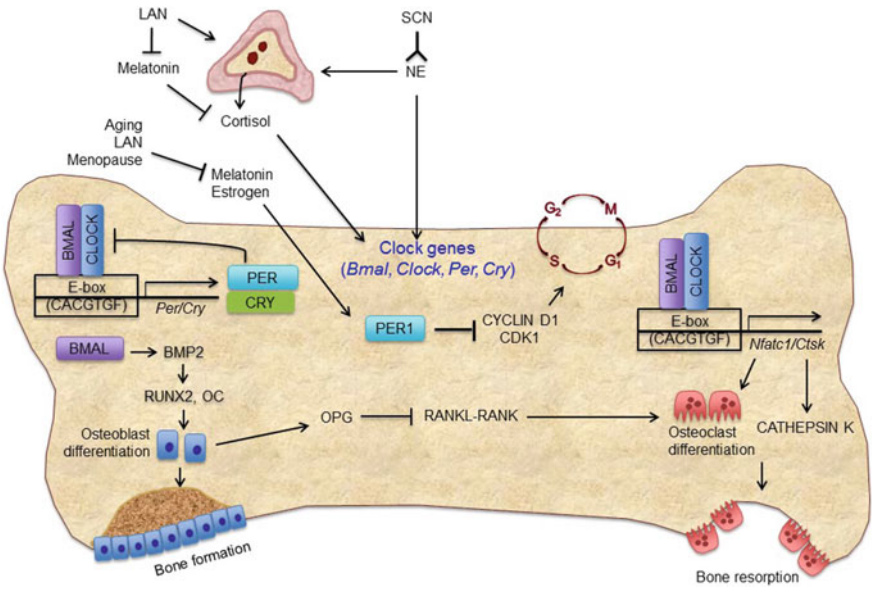

Fig. 3.2 Potential mechanisms underlying clock gene regulation of bone. As shown, clock genes, brain and muscle Arnt-like protein (Bmal), circadian locomotor output cycles kaput (Clock), Period (Per 1, 2, 3) and cryptochrome (Cry) are expressed in bone. BMAL:CLOCK heterodimers can regulate both Per/Cry expression in osteoblasts and Ctsk/Nfatc1 expression in osteoclasts by binding to E-box (CACGTGF)-containing motifs in their promoters. In osteoblasts, PER:CRY heterodimers can negatively regulate CLOCK:BMAL to decrease Per/Cry transcription. In osteoclasts, BMAL:CLOCK heterodimers can regulate Ctsk, which encodes CATHEPSIN K—a protein that dissolves bone and forms resorption pits and Nfatc1 expression, which is involved in osteoclast differentiation. BMAL can also induce BMP-2 expression resulting in expression of the osteogenic genes, Runx2, osteocalcin (OC). PER can also inhibit CYCLIN D1, which is involved in cell cycle progression through CDK1 at the G1/S interface. Both melatonin and estrogen can modulate PER expression and conditions that affect either melatonin levels (shift work, LAN, aging) or estrogen levels (menopause) could impact on circadian rhythmicity in bone. The control of bone through the SCN, though not essential, may occur through sympathetic regulation and humoral factors, specifically cortisol, from the adrenal glands to modulate clock gene expression. Cortisol was demonstrated to restore osteoblast-specific and osteoclast-specific genes in bone. Disruption of melatonin by LAN, aging or menopause could cause bone loss by inhibiting melatonin levels or increasing cortisol levels (Zvonic et al. 2007; Kondo and Togari 2015; Witt-Enderby et al. 2012; Min et al. 2016; Fu et al. 2005; Maronde et al. 2010; Samsa et al. 2016; Gery et al. 2007; Jung-Hynes et al. 2010)

particularly during the menopausal transition (Iguichi et al. 1982; Sack et al. 1986; Zhou et al. 2003). Besides age and menopause, LAN shifts both the phase of the melatonin rhythm as well as the production of melatonin through the pineal gland (Zeitzer et al. 2007; Kennaway et al. 2002; Blask et al. 2005).

The circadian variation of osteogenic genes was highest at the light/dark transition and during the hours of darkness when melatonin levels begin to rise and peak. In Witt-Enderby et al. (2012), increases in the clock gene Per2 and osteogenic

genes, Runx2, Bmp2, Bmp6, Bglap in mouse calvarial bone maintained under a 12:12 light:dark cycle were coincident with endogenous melatonin levels. This is consistent with the inducing effect of melatonin on osteoblasts (Roth et al. 1999; Sethi et al. 2010; Zhang et al. 2010; Park et al. 2011; Radio et al. 2006). Because melatonin levels can be inhibited by LAN, then disruptions in melatonin signaling through shift work or disease could lead to bone-related diseases. This is supported in studies where shift work increases fracture risk in nurses (Feskanich et al. 2009); adolescent idiopathic scoliosis is associated with impaired melatonin receptor signaling in osteoblasts (Sadat-Ali et al. 2000; Moreau et al. 2004; Azeddine et al. 2007; Yim et al. 2013); and children with Smith-Magenis syndrome have altered melatonin rhythms and bone abnormalities (Novakova et al. 2012; Potocki et al. 2000). Melatonin is also strongly involved with circadian entrainment of sleep/wake cycles and so disrupted melatonin levels resulting in poor sleep may also contribute to a loss of bone. This is demonstrated in postmenopausal women where BMD was correlated with sleep quality, that is, the poorer the sleep quality the lower the BMD (Wang et al. 2015). Because sleep quality correlated with BMD only in postmenopausal women and not in premenopausal women suggests that other factors like estrogen and, perhaps, melatonin may be involved. Both of these hormones decline during menopause as described previously.

The impact of melatonin deficiency on bone has been described previously. Low levels of melatonin alter bone morphology in fish (Fjelldal et al. 2004), chickens (Turgut et al. 2005; Kono et al. 2011), and mice (Oyama et al. 2006). Light exposure at night (LAN) or pinealectomy increases bone marker turnover (Ostrowska et al. 2003a, b) and decreases bone mass in rodent models (Machida et al. 1995; Fjelldal et al. 2004; Turgut et al. 2005; Oyama et al. 2006; Kono et al. 2011; Egermann et al. 2011). In humans, levels of melatonin are inversely correlated with levels of bone resorption markers in postmenopausal women (Ostrowska et al. 2001). Restoration of melatonin levels in vivo during the hours of darkness when melatonin peaks occur restores bone health (Ostrowska et al. 2003a, b) in rodents and in humans. In two clinical trials, Melatonin Osteoporosis Prevention Study, MOPS (Kotlarczyk et al. 2012) and Treatment of Osteopenia with Melatonin Study, MelaOst (Amstrup et al. 2015), nightly supplementation with melatonin renormalizes bone marker turnover in perimenopausal women (Kotlarczyk et al. 2012) and reverses bone loss in postmenopausal women with osteopenia, respectively (Amstrup et al. 2015).

3.3.2 Estrogen and Progesterone Effects on Bone and Bone Rhythms

Menopausal status influences diurnal rhythms of bone metabolism where the peaks in bone metabolism increase following menopause and even more so if a postmenopausal woman has osteoporosis described previously (Uebelhart et al. 1991;

Schlemmer et al. 1992; Greenspan et al. 1997; Eastell et al. 1992). The loss of the bone-protective hormones, estrogen, progesterone and melatonin, may be contributing to this since all three hormones decline as women transition through menopause (Iguichi et al. 1982; Sack et al. 1986; Zhou et al. 2003); and an exaggerated bone loss occurred in sheep when all three hormones were absent compared to a deficiency in either estrogen alone or melatonin alone (Egermann et al. 2011). The loss of estrogen, which inhibits osteoclasts (Srivastava et al. 2001; Saintier et al. 2006; Robinson et al. 2009), the loss of progesterone, which stimulates osteoblasts (Seifert-Klauss and Prior 2010) and the loss of melatonin, which both stimulates osteoblasts (Roth et al. 1999; Radio et al. 2006; Zaminy et al. 2008; Zhang et al. 2010; Sethi et al. 2010; Park et al. 2011) and inhibits osteoclasts (Suzuki and Hattori 2002; Koyama et al. 2002) may be exaggerating bone metabolism causing bones to weaken in women over time. Supplementation with estrogen and progesterone hormone therapy (HT) reduced the bone metabolism peaks back to premenopausal levels (Uebelhart et al. 1991) and supplementation with melatonin restored bone marker turnover in perimenopausal women (Kotlarczyk et al. 2012) and reversed bone loss in postmenopausal women with osteopenia (Amstrup et al. 2015).

Disruptions in melatonin signaling through LAN, work and sleep schedules and through the aging process, particularly menopause, could impact bone rhythms leading to bone loss (Maria and Witt-Enderby 2014). As mentioned previously, shift work (Feskanich et al. 2009; Quevedo and Zuniga 2010; Kim et al. 2013) and sleep (Wang et al. 2015) negatively impact bone, which probably is attributed more to disrupted clock rhythms and melatonin rhythms rather than sleep "per se" because these rhythms exist during the hours of darkness the time at which rodents are most active.

Circadian modulation of bone by melatonin and estrogen can be explained, in part, through their stimulatory effects on Per2 (Fig. 3.2). Per2 expression in mouse calvarial bone displayed a circadian rhythm where levels peaked during the hours of darkness when melatonin levels were highest. Coincident with high Per2 and melatonin levels increases in Runx2, Bglap, Bmp2 and Bmp-6 were also observed in mouse bone (Witt-Enderby et al. 2012). With respect to estrogen and Per2, Per2 was able to inhibit estrogen-inducible estrogen receptor alpha (ERα) expression and negatively regulates ER-mediated gene expression (Gery et al. 2007; Jung-Hynes et al. 2010). Appropriate levels of melatonin and estrogen would maintain Per2 expression in bone preventing excessive proliferation and a loss or disruption of the melatonin periodicity (through LAN, shift work, menopause, or aging) or estrogen through menopause would disrupt Per2 rhythms and/or decrease Per2 levels producing excessive proliferation in bone resulting in increases in bone density and bone volume.

3.3.3 Cortisol Effects on Bone and Bone Rhythms

3.3.3.1 Circadian Variation of Cortisol and Glucocorticoid Signaling in Bone

Glucocorticoids play important roles in mediating the circadian output from the SCN. Under normal non-stressed conditions, both humans and rodents display rhythmic patterns of cortisol or corticosterone secretion from the adrenal cortex, respectively, that are maintained by the SCN. In humans, plasma cortisol rhythms rise within 30 min of waking and gradually decline throughout the day until reaching their nadir around midnight. Rodent corticosterone levels peak between ZT8 and ZT12 (end of light cycle) and reach their nadir at ZT 20 (end of night cycle) (Zvonic et al. 2006; Ptitsyn et al. 2006; Ishida et al. 2005; Chung et al. 2011). Even though human cortisol peaks are out of phase with rodents by about 12 h, both levels rise prior to or at the onset of the active phase—for nocturnal animals this would be the onset of darkness—to prepare the body for waking and activity. Coincident with the rise and fall of circulating plasma glucocorticoid levels is the responsiveness of tissues and cells to cortisol or corticosterone in humans and rodent, respectively, due to rhythmic repression of the glucocorticoid receptor (Bedrosian et al. 2016). This is demonstrated in mouse calvarial bone, where the genes involved in the glucocorticoid receptor signaling pathway (e.g., glucocorticoid receptor, heat shock proteins 70 and 90) displayed circadian variation (Zvonic et al. 2007).

3.3.3.2 Cortisol Effects on Clock Genes Expressed in Osteoblasts and Osteoclasts

In Kondo and Togari (2015), mPer1 expression in the SCN displayed a 4 h phase-advanced circadian profile compared to mPer1 expression in bone suggesting that the rhythmic expression of mPer1 in mouse SCN is regulated directly by master clock genes, while systemic signals modulated by the SCN govern the peripheral rhythms in bone. The involvement of humoral factors in regulating clock gene rhythms in bone cells was tested using human osteoblasts (Sam-1 cells) first using high serum concentrations and then using isoproterenol, a sympathomimetic agent and dexamethasone, a glucocorticoid. Here it was demonstrated that hPER1 and hBMAL expression in Sam-1 cells treated with 50% fetal bovine serum for 2 h followed by serum starvation or following exposure to isoproterenol or dexamethasone was induced (Komoto et al. 2012). These isoproterenol- or dexamethasone-induced effects on clock genes expressed in bone also induced the circadian expression of hCOL and hALP—two osteoblast differentiation marker genes—and *mCtsk* and *Nfatc1*—two osteoclastic genes involved in bone resorption or osteoclast differentiation. Glucocorticoid involvement in bone circadian rhythms was further assessed using adrenalectomized mice maintained on a 12:12 light:dark

cycle. In these mice, the rhythms of mCtsk and Nfatc1 disappeared; however, the circadian rhythms of mPer1 and mBmal persisted. Injection with dexamethasone at 7 pm, the time when cortisol levels begin to rise in intact mice, restored the circadian rhythm of mCtsk. These data support the idea that circadian clocks in the SCN, through the sympathetic nervous system and humoral routes, can communicate with bone cells to synchronize them with standard circadian time (Oishi et al. 2005; Silver et al. 1996; Inouye and Kawamura 1979).

3.3.3.3 Effect of Lifestyle, Light Exposure and Environment on Cortisol Levels

Many variables can disrupt glucocorticoid rhythms as reviewed (Bedrosian et al. 2016). For example, exposure to light pulses can both phase shift and alter the amplitude of cortisol rhythms. Workplace lighting, especially in places with low light levels due to the absence of windows, can elevate nighttime cortisol concentrations. Shift work is also associated with disrupted cortisol rhythms where levels in night shift workers are higher during the day and lower during the night compared to levels in day shift workers (Bedrosian et al. 2016). Surgeons on call experience suppressed morning cortisol levels compared to pre- and post-call salivary cortisol levels. Elevated cortisol levels are typically observed in individuals who are sleep deprived or who experience social jet lag (Bedrosian et al. 2016).

Changes in the diurnal variation of cortisol may be attributed to melatonin as LAN, sleep, and shift work can also regulate melatonin levels. Melatonin can modulate cortisol levels (Ogle and Kitay 1978) where its effects are dependent on age (Cagnacci et al. 1995) gender (Lesniewska et al. 1990; Cagnacci et al. 1995), menopausal status, estrogen status (Cagnacci et al. 1997) and the timing of exposure (Cagnacci et al. 1995, 1997). Melatonin does produce direct inhibitory actions on adrenalcortical cells (Sewerynek and Lewinski 1989; Richter et al. 2008) and this effect is most sensitive during hours of darkness (nighttime) due to higher expression of MT1 melatonin receptors expressed on adrenal cortical membranes (Richter et al. 2008). Therefore, decreases in melatonin levels through LAN could modify the circadian rhythmicity of bone metabolism by decreasing Per2 and/or increasing cortisol levels described in Fig. 3.3.

3.4 Strategies to Prevent Bone Loss Caused by Circadian Disruption

Many variables cause circadian disruption—some we can control and some that we cannot. The aging process cannot be stopped, society will always need shift workers and light pollution is part of our world—not just in urban societies. So, how can humans minimize bone loss caused by circadian disruption? The following

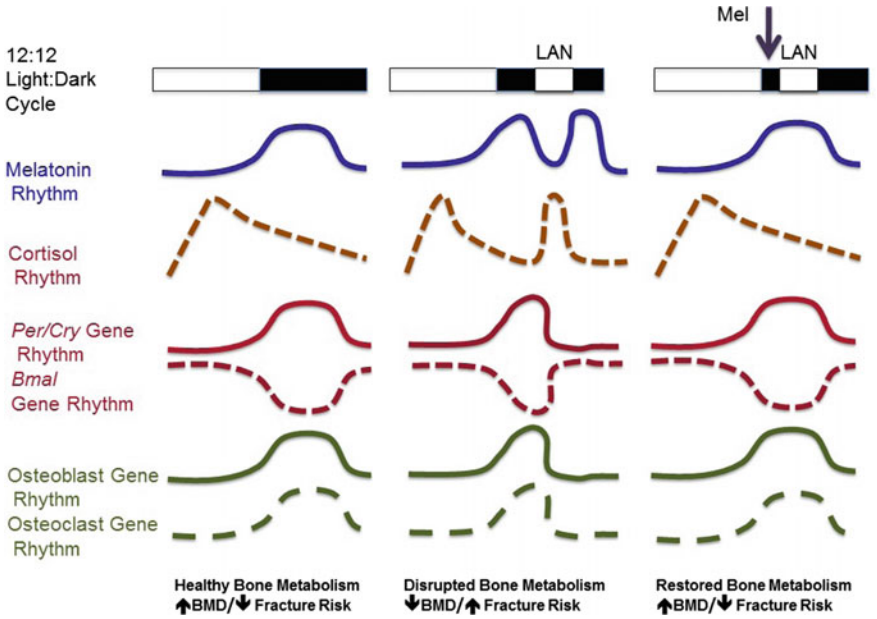

Fig. 3.3 Relationship between light:dark cycle, melatonin and cortisol rhythms on clock gene, osteoblastic and osteoclastic gene expression. Under conditions that maintain healthy bone metabolism resulting in high density bone with low fracture risk, melatonin levels rise during the dark cycle and fall during the light cycle (*white boxes* indicate 12 h of light and *black boxes* indicate 12 h of darkness). Cortisol levels are opposite to melatonin levels where peaks occur right at the onset of waking and gradually decline throughout the day reaching their lowest levels at midnight. The circadian variation of the clock genes, Per and Cry, and osteogenic genes, Runx2, Bmp2, Bmp6, Bglap are coincident with endogenous melatonin levels with their levels being highest at the light/dark transition and during the hours of darkness when melatonin levels begin to rise and peak. Bmal is anti-phase to Per where its levels are highest during the hours of light and lowest during the hours of darkness. Light exposure at night (*LAN*) decreases melatonin levels and disrupts its rhythm which leads to rises in cortisol levels during the night; disrupted clock rhythms (changes in amplitude or timing of peaks); and disrupted in osteoblast and osteoclast rhythms (changes in peaks and rhythms). This leads to changes in bone metabolism rhythms resulting in low density bone with increased fracture risk. Restoration of melatonin peaks by melatonin supplementation during LAN restores the melatonin, cortisol, clock and bone rhythms back to normal (Zvonic et al. 2007; Kondo and Togari 2015; Witt-Enderby et al. 2012; Min et al. 2016; Fu et al. 2005; Maronde et al. 2010; Samsa et al. 2016; Gery et al. 2007; Komoto et al. 2012; Oishi et al. 2005; Silver et al. 1996; Inouye and Kawamura 1979; Jung-Hynes et al. 2010)

simple pearls of wisdom may help and are available to all regardless of socio-economic status, geographical location, health status, gender and age:

Turn down light exposure at night to get better sleep. Our skies are becoming brighter. In 2001, 62% of the world's population was living under sky brightness higher than baseline levels; in the United States and Europe, these numbers were as high as 99% (Bonmati-Carrion et al. 2014). Also, the majority of people living in the United States (over four fifths) and the European Union (two-thirds) regularly

experience sky brightness greater than the light reflected from a full moon (Navara and Nelson 2007). Since the 1960s, artificial lighting has moved away from an incandescent-bulb form, which consists of mainly low-level yellow wavelengths to higher intensity discharge lamps consisting mainly of blue wavelengths (Bonmati-Carrion et al. 2014). Blue/violet wavelengths (459 nm) can lower circulating melatonin levels through their effects on retinal ganglion cells responsible for detecting light and suppressing melatonin production in humans (Navara and Nelson 2007). It is estimated that 90% of adults use some form of electronic device within one hour of bedtime. In fact, melatonin levels can be suppressed by half following exposure to a low-level incandescent bulb for only 39 min (Navara and Nelson 2007) and use of a tablet computer or e-reader before bed is sufficient to suppress melatonin (Bedrosian et al. 2016). Even nighttime lights in our streets and cities are clearly linked with modifications in human sleep behaviors and also impinge on the daytime functioning of individuals living in areas with greater outside light (Ohayon and Milesi 2016). Minimize LAN by wearing an eye mask during sleep, or closing the shades to reduce light from outside, or limiting the use of electronic devices before bed; or by using applications that filter blue light from these electronic devices. The accumulated effect of minimal LAN may reduce the risk of osteopenia and osteoporosis.

Increase your daytime exposure to light. Daytime sunlight is necessary for vitamin D synthesis, the active metabolites of which are critical to maintaining healthy bone. Numerous studies demonstrate that melatonin and vitamin D complement one another and regulate similar processes of cells, tissues, organs, and systems, such that deficiencies of either one can result in similar effects on health and well-being (Smolensky et al. 2015), including bone (Melatonin-micronutrients Osteopenia Treatment Study: NCT01870115). According to Canadian and US population-based surveys, people spend $\sim$12% of their time outdoors, with half of this amount spent inside vehicles. On average, people spend between 1 and 2.4 h per day outside during the summer months, which drops to 0.4–1.3 h per day during the winter months no matter where one lives (Cole et al. 1995; Hubert et al. 1998; Diffey 2011). By increasing time spent in natural lighting, bone loss may be prevented by increasing calcitriol, the active form of vitamin D3, and by realigning bone circadian rhythms with the endogenous clock, with melatonin and cortisol rhythms.

Modify Your Diet—especially when you eat. A rise in North American fast-food profits were observed during the late night and early morning hours starting in the 1990s (MSNBC.com 2004). Diet, the timing of meals and alcohol ingestion can alter one's circadian rhythms in both a light-dependent and -independent manner. Alcohol ingestion may result in lowered melatonin levels (Navara and Nelson 2007; Stevens 2006) which, in turn, could alter estrogen levels leading to bone loss as described in Figs. 3.2 and 3.3. Eat consistently during the day and try to finish your final meal two hours before bedtime. Limit alcohol and coffee consumption.

Reduce stress and supplement with nocturnal melatonin. The deleterious effects of circadian disruption on bone could be minimized through countermeasures like realigning one's circadian rhythm of bone metabolism with their melatonin levels by taking melatonin supplementation at night (Fig. 3.3). The use of melatonin during the hours of darkness would serve many purposes. First, it would provide a bone-enhancing effect through its actions on osteoblasts and inhibitory effect on osteoclasts directly (Kotlarczyk et al. 2012; Amstrup et al. 2015; Radio et al. 2006; Sethi et al. 2010). Melatonin has been shown to have equal efficacy on increasing bone density in rodents as a one year therapeutically relevant dose of estrogen-progesterone HT (0.5 mg E_2/50 mgP_4) given for the same period of time (Witt-Enderby et al. 2012). This is important because even though HTs are commonly used for their bone-protective functions through estrogen's inhibitory actions on osteoclasts (Srivastava et al. 2001; Saintier et al. 2006; Robinson et al. 2009) and progesterone's stimulatory effects on osteoblasts (Seifert-Klauss and Prior 2010), many women and certainly not men would not find HT a viable option for the prevention of bone loss through circadian disruption due to untoward side effects. Second, it would regulate sleep rhythms, which also have been shown to modulate bone density (Wang et al. 2015). Third, it would realign Per2 rhythms to maintain appropriate proliferative states of bone (Fig. 3.2). Fourth, it would resynchronize the peripheral clocks in bone with the master circadian regulator, the SCN, and minimize the release of humoral factors known to increase bone metabolism like sympathomimetic agents and cortisol (Fig. 3.3).

Overall, advanced technology has brought the world closer through the use of computers and smart phones. In seconds and by a simple click of the computer key or by pressing a button on one's smart phone, information can be delivered in seconds. Even though technology has eased some of life's burden in modern society, it has brought other challenges that are adversely impacting on the bone health of our people, specifically with managing time, getting enough sleep and eating well. Lifestyle changes that include getting sleep in complete darkness, reducing use of electronic devices before bed, getting more light exposure during the day (particularly natural lighting from the outdoors) and eating meals at regular times, as basic as these seem, would help realign one's bone rhythms with the light/dark cycle and their endogenous clock. Over time, this would reduce the risk of bone loss preventing osteopenia, osteoporosis and fracture by restoring and maintaining healthy nightly melatonin peaks while minimizing cortisol peaks. Considering that nine million adults in the United States suffer from osteoporosis and fifty million from osteopenia now, which is predicted to increase by 2.9 million for osteoporosis and 14.3 million for osteopenia by 2030 (Maria and Witt-Enderby 2014), making these minor lifestyle adjustments now could significantly improve the health of our society.

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Chapter 4
Aging and the Circadian Control of the Gastrointestinal System: From the Brain to the Gut Microbiome (and Back)

Vincent M. Cassone, Jiffin K. Paulose and Clifford E. Harpole

Abstract The effect of aging on circadian clocks within the gastrointestinal system and the role played by melatonin and circadian clocks in the process of gastrointestinal aging is reviewed. Although we know quite a lot about the physiological and molecular mechanisms of circadian clocks in mammals, we know very little about the mechanisms of food-entrainable rhythmicity. The role played by the pineal hormone melatonin has been posited as a key to understanding aging and clock function, but the evidence is incomplete. Finally, new data about aging and circadian control of the intestinal microbiome is placed in the context of the circadian system as a whole.

Keywords Circadian · Food entrainable oscillator · Gastrointestinal · Light entrainable oscillator · Melatonin · Microbiome

4.1 Introduction

The process of normal aging can have moderate to severe effects on gastrointestinal (GI) function. Esophageal contractions and tension decreases (Geokas and Haverback 1969), the stomach loses much of its elasticity and is less resistant to damage (Geokas et al. 1985), and the intestinal lumen can show marked changes in the microflora (Thomson and Keelan 1986). In addition, pancreatic and hepatic fibrosis increases as we age (Geokas et al. 1985). Further, other, indirect or secondary factors associated with age can also profoundly upset gastrointestinal homeostasis, including a decrease in physical activity, an increase in the use of several drugs, especially non-steroidal anti-inflammatory drugs (NSAIDS), which can cause GI bleeding, and opiates, which result in constipation (Bitar et al. 2011).

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Circadian Organization

One of the "indirect" factors that may affect the process of gastrointestinal health during aging is the circadian system, or clock, which is a fundamental property of most living organisms on Earth (Bell-Pedersen et al. 2005). In animals, circadian rhythms are easily observed by monitoring locomotor behavior, such that diurnal animals are active during the light portion of the light:dark cycle (LD), the day, while nocturnal animals are active during the dark, the night. In order to establish that a process is *circadian*, rather than merely diurnal or nocturnal, experimenters must place the animal in constant environmental lighting, either constant darkness (DD) or constant dim light (dimLL; bright constant light, LL, has other deleterious effects on clock expression). In DD, animals express internally generated circadian rhythms of behavior and physiology such that the period of the rhythm is not exactly 24 h. Under these circumstances, the concepts of day and night are subjective, relative to the animal's experience rather than to the external world. Hence, a nocturnal animal is active during the *subjective night,* while a diurnal animal is active during the *subjective day.* These internally generated rhythms are synchronized to local time via *entrainment*.

The mammalian circadian system is organized hierarchically, with a "master" pacemaker in the hypothalamic suprachiasmatic nuclei (SCN), which is entrained to LD cycles via the retinohypothalamic tract and is therefore a "light-entrainable oscillator" (LEO) (Moore 2013). The evidence for the preeminence of the SCN in circadian organization is undeniable. First, surgical destruction of the SCN abolishes the expression of overt circadian rhythms in locomotor behavior, sleep, body temperature, many endocrine signals and other physiological processes (Moore 2013). Secondly, the SCN express circadian rhythms of electrical, metabolic, and molecular activity in vivo and in vitro (Gillette and Tischkau 1999; Moore 2013), and, thirdly, transplantation of embryonic SCN tissue or cells into the site of an SCN lesion restores circadian rhythmicity to arrhythmic, SCN-lesioned rodents (Aguilar-Roblero et al. 1986; Ralph et al. 1990; Earnest et al. 1999). Thus, in mammals, the oscillators within the SCN are master pacemakers that control the circadian patterns of most, if not all, behavioral, physiological and molecular processes downstream.

Yet, there is an equally formidable body of evidence for SCN-independent peripheral oscillations (Escobar et al. 2009; Pezuk et al. 2010; Mohawk et al. 2012). At the early outset of research on SCN function, one of the discoverers of the SCN, Friedrich Stephan at Florida State University, noted that rats could be entrained to a timed meal and that SCN-lesioned rats were still capable of predicting a meal on a daily basis (Davidson and Stephan 1999; Stephan 2002; Cassone and Stephan 2002). Many researchers have looked for "the" food-entrainable oscillator (FEO) with negligible success. Since the phenomenon was first observed as a behavioral rhythm, several scientists looked for central nervous system (CNS) sites for the FEO; however, these results have been controversial. In a very high profile study, several authors (Gooley et al. 2006; Fuller et al. 2008) have pointed to the hypothalamic dorsomedial nucleus (DMN) as the site for food entrainment. However, other researchers (Landry et al. 2006; Mistlberger et al. 2008) showed

that DMN lesions do not abolish food anticipatory behavior or entrainment to a timed meal and have questioned these results, although Mistlberger acknowledged later that the DMN may be involved in food anticipatory behavior (Mistlberger 2011). Conversely, Davidson et al. (2000) found that food anticipatory behavior was reduced or abolished by destruction of the parabrachial nucleus (PBN). However, since this structure also conveys sensory input from the periphery, the PBN may not be the seat of FEO oscillation but rather a conduit for the Zeitgeber's influence on central oscillators. It is also possible that the actual FEO is within the GI tract itself (Comperatore and Stephan 1987; Davidson et al. 2003; see below). CNS sites must at some point be involved in FEO activity, but its/their location is at this stage unverified. Perhaps, as is suggested by Verwey and Amir (2009), "a distributed neural system underlies…food anticipatory activities."

At the molecular level, vertebrate circadian rhythms are regulated by a highly conserved set of genes, collectively called "clock genes", whose products dynamically interact to elicit rhythmic patterns of transcription, translation, biochemical and physiological processes, and behavior (Bell-Pedersen et al. 2005; Mohawk et al. 2012). The central core of this gene network can be broadly characterized as "positive elements" *clock* and *bmal1,* and "negative elements" *period 1 (per1), period 2 (per2), period 3 (per3)* and the cryptochromes *cryptochrome 1 (cry1)* and *cryptochrome 2 (cry2)* (Fig. 4.1). *Clock* and *bmal1* are transcribed and translated in the cytoplasm, where their proteins, CLOCK and BMAL1, dimerize and reenter the nucleus and activate transcription of the negative elements. The *pers* and *crys* in turn are transcribed, translated in the cytoplasm, and their proteins, the PERs and CRYs, form oligomers with Casein kinase 1ε (CK1ε), that reenter the nucleus to interfere with the CLOCK/BMAL1-mediated activation. CK1ε phosphorylates PER 1, 2 and 3, delaying its nuclear translocation and thereby extending the circadian period. This circadian period, generated by the transcription translation feedback loop (TTFL), in turn, must be entrained to an external cue (or *Zeitgeber)*, to be adaptive. Typically, circadian systems entrain to the environmental LD cycle (LEO). This will be referred to below as the light-entrainable oscillator (LEO) associated with the TTFL (LEO/TTFL). There are multiple other components of this gene network, but space prevents detailed description, and it has been exhaustively reviewed elsewhere (Albrecht 2004; Bell-Pedersen et al. 2005; Roenneberg and Merrow 2003).

Interestingly, food entrainment and anticipatory behavior do not rely on the expression of canonical clock genes. Mice deficient in *bmal1*, which are arrhythmic when they are placed in constant darkness (DD), exhibit robust food anticipatory activity (Pendergast et al. 2009; Mistlberger et al. 2009; Storch and Weitz 2009). Similarly, mice with double knockout of *per1* and *per2* entrain to timed meals and exhibit food-anticipatory behavior (Storch and Weitz 2009). Thus, the food-entrainable oscillator can anticipate and entrain to a timed meal independently of both the SCN and the canonical clock gene network; an intact molecular LEO/TTFL is not necessary for food entrainment (Fig. 4.1).

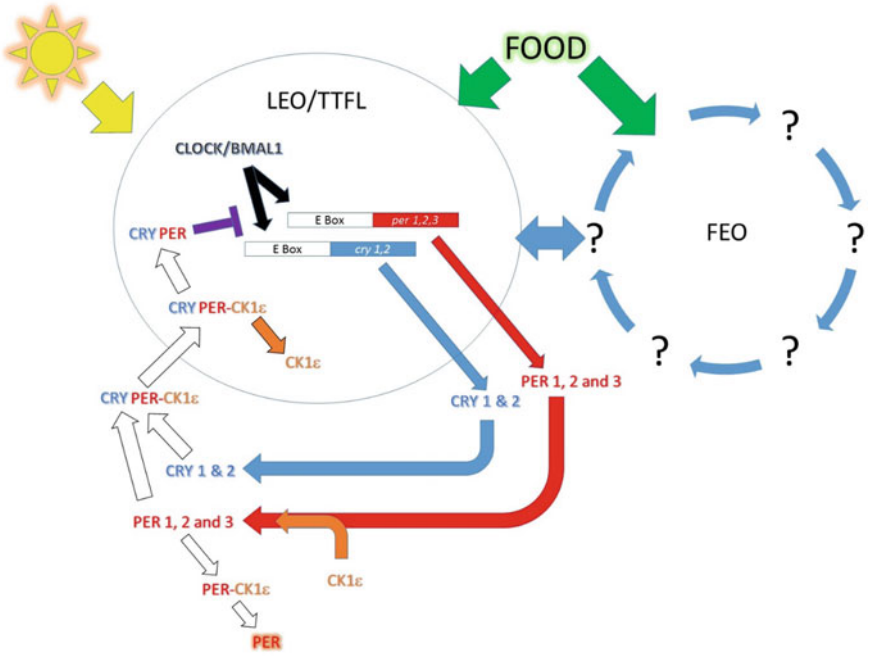


Fig. 4.1 Schematic of the molecular light-entrainable (LEO) transcription-translation feedback loop (*TTFL*) for circadian rhythm generation, about which we know quite a bit, and food entrainable oscillator (*FEO*), about which we know very little. Positive elements CLOCK and BMAL1 stimulate transcription of genes whose promoters contain E-boxes, including negative elements *period 1, 2 and 3 (per 1,2,3)* and *cryptochromes 1 and 2 (cry 1,2).* These are translated in the cytoplasm and form oligomers with *casein kinase 1ε (CK1ε). CK1ε* phosphorylates the PERIOD proteins, targeting them for proteolysis, slowing the rate of nuclear transport. Once inside the nucleus, the CRY/PER dimers interfere with the CLOCK/BMAL1 transcriptional activation. Both food and light can entrain the LEO/TTFL, but it is not necessary for food entrainment, since clock gene knockout does not eliminate food anticipatory behavior or entrainment in rodents. The molecular mechanism for this FEO is not known

4.2 Peripheral Circadian Clocks and the Gastrointestinal System

As one might expect, clock genes are intensely and rhythmically expressed within the SCN (Reppert and Weaver 2002). However, these genes are expressed in multiple other tissues ranging from muscle to liver to fat (Abe et al. 2002). Indeed, nearly every tissue in the mammalian body expresses clock genes in a circadian fashion. For example, using a knock-in mouse strain carrying the gene for *per2* fused with firefly luciferase (PER2::LUC; Yamazaki and Takahashi 2005), in which bioluminescence is induced when *per2* is transcribed and translated, Yoo et al. (2004) observed rhythms of bioluminescence emanating from the cornea, lungs,

liver, kidney, pituitary and tail, as well as some CNS sites such as SCN. These rhythms persisted in tissues taken from SCN-lesioned mice, but the relative phases of these rhythms were more variable, if not completely random. Several authors have observed similar rhythms in many other tissues (Yamazaki and Takahashi 2005). The emerging view is that peripheral oscillators are present throughout the body but are synchronized by the pacemakers within the SCN.

Among the peripheral tissues that express clock gene rhythms is the gastrointestinal system, from the pharyngeal mucosa to the rectum and many other associated GI tissues, such as the liver, the exocrine pancreas and salivary glands (Davidson et al. 2003; Polidarova et al. 2009; Hoogerwerf 2010; Hoogerwerf et al. 2007, 2008, 2010). Mouse stool number and weight are rhythmically produced in vivo such that feces is produced predominantly during the night in mice held in light: dark cycles (LD) and during the subjective night (when they are active) in mice maintained in constant darkness (DD) (Hoogerwerf et al. 2007; Malloy et al. 2012), and measures of colonic motility in vivo using an ambulatory telemetry system exhibits slow wave rhythms of colonic pressure during the day and subjective day that transitions into higher frequency contractions during the night and subjective night (Hoogerwerf et al. 2007, 2010). In addition, electrolyte absorption by the colon is regulated by a circadian clock in vivo (Sotak et al. 2011). In vitro, colonic tissue contractility in response to varying concentrations of acetylcholine (Ach) is also rhythmic, such that colonic circular muscle contractility in response to Ach is greater during the night and subjective night than during the day and subjective day (Hoogerwerf et al. 2007).

The mouse gastrointestinal system expresses clock genes in a rhythmic fashion both in vivo and in vitro (Hoogerwerf et al. 2007). Gastrointestinal tissues such as the stomach, proximal, middle and distal colonic tissues express *clock, bmal1, per1, per2, per3, cry1* and *cry2* mRNA (Fig. 4.2), as well as BMAL1, PER1 and PER2 protein. Staining is most intense in epithelial cells along colonic crypts, especially in the bases of the crypts, and is also present in the myenteric plexus of the stomach and throughout the colon (Hoogerwerf et al. 2007). Similarly, Sladek et al. (2007) have shown that rat colonic epithelial cells rhythmically express clock genes as well (Sladek et al. 2007). Clock gene rhythms in the rat gastrointestinal epithelium exhibits a "temporal gradient" such that the upper GI tract is phase advanced relative to the lower GI cells (Polidarova et al. 2009).

These GI rhythms are regulated by the clock gene network, since double knockout of *per1* and *per2* abolish circadian rhythms of stool production as well as measures of intestinal motility in vivo and in vitro (Hoogerwerf et al. 2010). However, the SCN still synchronizes these rhythms, since SCN lesion of mice fed *ad libitum* abolishes circadian rhythms of stool output (Malloy et al. 2012). The SCN appears to synchronize the GI rhythms through circadian control of sympathetic tone, since chemical sympathectomy with the neurotoxin 6-hydroxydopamine (6OHDA) also abolishes rhythms in stool output of mice in which food is constantly available, and pulsed administration of the β-adrenergic agonist isoproterenol to cultured colons carrying the PER2::LUC knock-in phase shifts rhythms of bioluminescence (Malloy et al. 2012). Importantly, timed feeding

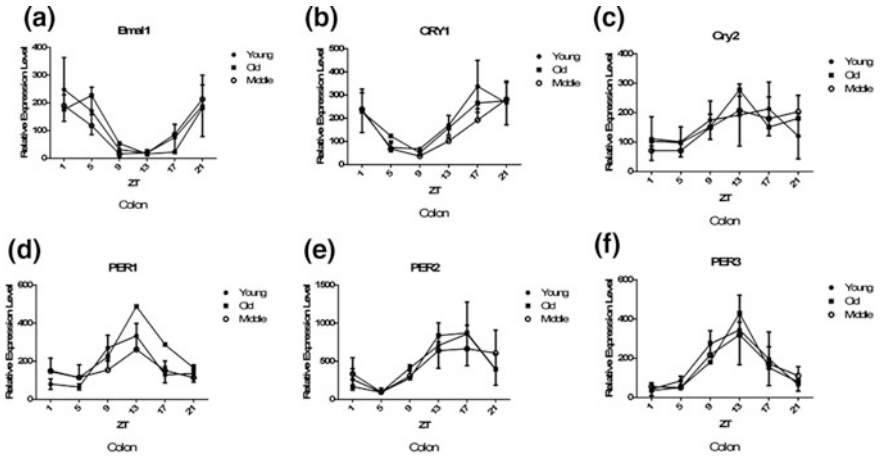


Fig. 4.2 Age does not affect daily changes in colonic clock gene content. Rhythms of *Bmal1* (**a**), *cry1* (**b**), *cry2* (**c**), *per1* (**d**), *per2* (**e**) and *per3* (**f**) are similar in young (2 month old), middle-aged (12 month old) and aged (24 month) old CBA mice

overcomes these manipulations, since SCN-lesioned and 6OHDA-treated mice still anticipate a timed feeding (Malloy et al. 2012).

Interestingly, timed feeding regimes in which food is present during the subjective day, when mice normally do not eat, phase-shift rhythmic clock (*bmal1* and *per2)* mRNA and protein 12 h, while expression of *bmal1* and *per2* mRNA in the SCN is unaffected by the restricted feeding (Hoogerwerf et al. 2007). This led us to believe that the gastrointestinal tract contains all the machinery for rhythmicity and to anticipate a novel food presentation. Finally, cultured intestinal tissues from transgenic mice carrying PER2::LUC exhibit robust circadian rhythmicity, punctuating the view that the mouse gastrointestinal system contains a circadian clock (Davidson et al. 2003; Malloy et al. 2012). Similarly, Polidarová et al. (2011) have shown that rats exposed to constant light (LL) for 30 days and fed *ad libitum* became arrhythmic in locomotor activity, as is typical for rats and many circadian systems. Expression profiles of clock genes and several clock-controlled genes in liver, duodenum and colon also became arrhythmic. Restricted feeding restored clock gene rhythms in the liver and duodenum, but the colon's rhythmicity was only partially restored. Thus, even if the gastrointestinal tract is not "the" food-entrainable oscillator, it certainly is "a" food-entrainable oscillator.

Even so, in view of the fact that neither the SCN nor clock genes are necessary for food entrainment, it is still an open question whether the orchestration of circadian orchestration represents an orchestration of peripheral clock genes by the SCN. However, this is NOT to say that the SCN and clock genes are not sufficient for such orchestration. Clearly, more objective research is required at a systems-level to begin answering these questions. Having said this, the role of clock genes and circadian organization is at least strongly linked to the processes described below.

4.3 Aging and Circadian Clocks

Aging affects circadian clock function (Froy and Miskin 2007). During normal aging, the amplitude of circadian rhythms within the human SCN is reduced (Hofman 2000). Daily patterns of SCN vasopressin (AVP) content in the SCN in brains of aged human cadavers, who died at different times of day, show diminished amplitude compared to the AVP content in the SCN taken from brains of younger human decedents (Hofman 2000; Hofman and Swaab 2006), and the circadian patterns of core body temperature and several endocrine signals are disrupted or lost during the aging of normal human volunteers (Hofman and Swaab 2006). Aged rodents such as mice, rats and hamsters exhibit fragmented patterns of wheel-running activity in DD as compared to young rodents, and age decreases the ability of rodents to re-entrain to new photoperiods in a simulated jetlag regime (Scarbrough et al. 1997; Valentinuzzi et al. 1997; Davidson et al. 2008). In vitro, the SCN of transgenic rats with a luciferase reporter indicating *per1* expression show persistent rhythms of *per1* expression that exhibit a shortened circadian period and decreased amplitude of aged versus younger rats (Yamazaki et al. 2002).

There are remarkably few reported effects of aging on the gastrointestinal clock, but there are several studies that have looked at circadian patterns in other peripheral tissues during aging. Yamazaki et al. (2002) showed that circadian rhythms of *per1* bioluminescence in several brain and peripheral tissues of rats, including retrochiasmatic area (RCA), paraventricular nucleus of the hypothalamus (PVN), pineal gland, pituitary and arcuate nucleus in the brain, as well as cornea, kidney, liver and lung, were largely unaffected by age, although RCA and PVN were phase-advanced in older rats. In contrast, Claustrat et al. (2005) found that the amplitude of rhythms in *per1-3* expression was reduced in liver and heart of aged mice, while *bmal1* expression was increased.

Preliminary data from our laboratory shows very little to no effect of aging on central clock gene expression in either small intestine or colon (Fig. 4.2). Young (2 months), middle-aged (12 months) and extremely aged (24 months) CBA mice were sacrificed at 4 h intervals in a LD14:10 light: dark cycle, and various peripheral tissues were collected for nanoString nCounter analysis of 40 different clock and clock-controlled genes. While daily patterns of the core, canonical clock genes were expressed rhythmically with appropriate phase relations, there was no systematic effect of age on the temporal pattern of clock gene expression, even in the extremely aged mice (Fig. 4.2). Thus, while the SCN and behavioral outputs of the circadian system are affected by age, the central gastrointestinal clock is relatively immune to the onslaughts associated with aging.

This is not to say that gastrointestinal clock outputs are not affected by age. Even so, the evidence is limited. For example, we have found that neuronal nitric oxide synthase (nNOS) is a clock-controlled gene in the mouse colon (Hoogerwerf et al. 2008) and that circadian rhythms of intestinal contractility and defecation are abolished in $nNOS^{-/-}$ knockout mice (Hoogerwerf et al. 2010). In Wistar rats, Vinod and Jagota (2016) report that nitric oxide levels as measured by Griess assays

are rhythmic on a daily basis with higher levels expressed during the night than during the day. These authors claim that the amplitude of the NO rhythm declines in aged 26 month old rats. These observations are admittedly from different species, but they raise the possibility that, even though the central clock mechanism in the gastrointestinal tract is only marginally affected, the aging of its rhythmic outputs may deleteriously affect physiological gastrointestinal rhythms.

4.4 Gastrointestinal Melatonin

Melatonin has also been implicated in aging as well as in gastrointestinal health. The mammalian pineal gland synthesizes and secretes melatonin on a daily and circadian basis such that blood, brain and tissue levels of the hormone are elevated during the night and subjective night (Zawilska and Nowak 1999). Pinealocytes and retinal photoreceptors take up the amino acid tryptophan (Trp) and convert Trp to 5-hydroxytryptophan (5-HTrp) via tryptophan hydroxylase (TPH). Then, aromatic amino acid decarboxylase (AAADC) converts 5-HTrp to serotonin (5HT). During the night, arylalkylamine-N-acetyltransferase (AANAT) converts 5HT to N-acetylserotonin (NAS), which is in turn converted to melatonin by hydroxyindole-O-methyl transferase (HIOMT). In mammals, this rhythm is under the direct control of pacemakers within the SCN via a multi-synaptic pathway that includes the sympathetic nervous system. In humans, there is a dramatic decrease in the amplitude of serum melatonin levels as they age from 21–25 years of age to 82–86 years of age (Waldhauser et al. 1998; Bubenik and Konturek 2011). The most dramatic decrease typically occurs between the ages of 50–60. These decreases in amplitude have been suggested to contribute to aging itself through a decrease in purported antioxidant properties of the hormone (Reiter 1995, 1997), either through receptor mediated actions or through direct chemical hydroxyl free radical scavenging properties of the hormone.

Interestingly, melatonin is also synthesized within enterochromaffin and/or enterochromaffin-like cells in the gastrointestinal system (Raikhlin et al. 1975; Raikhlin and Kvetnoy 1976; Quay 1976). The evidence for this is primarily from immunocytochemical detection of melatonin itself in these cells, in which estimates of gastrointestinal melatonin levels range from 10 to 400X serum levels of the hormone (Bubenik 1980; Huether 1994). However, there is also gene expression data that shows expression of TPH, DDC, AANAT and HIOMT (ASMT) in small intestine, colon and pancreas, as well as both melatonin receptors (MT1 and MT2) (Soderquist et al. 2015), so it is likely that the biosynthetic pathway for gastrointestinal melatonin is similar, if not identical, to pineal and retinal melatonin biosynthesis. Furthermore, Diss et al. (2013) have shown that melatonin is released from cultured distal colon of the mouse, punctuating the view that the gastrointestinal tract does indeed synthesize and release melatonin. Remarkably, in spite of the fact that the gastrointestinal system synthesizes melatonin (Huether 1994),

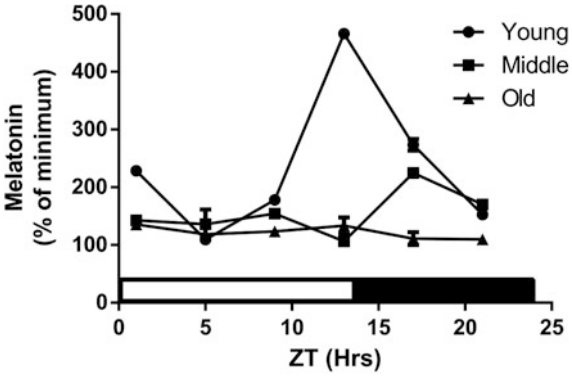


Fig. 4.3 Fecal melatonin content is dramatically affected by age in CBA mice. Young 2 month old mice exhibit a high amplitude rhythm that peaks at lights off. Middle-aged 12 month old mice exhibit a nocturnal rhythm of fecal melatonin content. The rhythm is abolished in aged 24 month mice

gastrointestinal melatonin does not pass into the systemic circulation (Bubenik 1980; Konturek et al. 2011). It is therefore an open question as to what exactly does melatonin do in the gastrointestinal tract.

Several authors have suggested that gastrointestinal melatonin affects gastrointestinal function itself, preventing mucosal ulcerations, reduction of gastric release of hydrochloric acid, and stimulating the gastrointestinal immune system (Bubenik 2002; Kvetnoy et al. 2002; Bubenik and Konturek 2011). Further, Bubenik (2002) reported that melatonin is also secreted into the lumen of the gastrointestinal system, either via the hepatic duct or from the enterochromaffin cells themselves. If this is the case, one would expect that melatonin should be detected in the feces. To answer this question, we collected feces every 4 h over 24 h in a light: dark cycle of LD 14:10 from male CBA mice of three different ages: young (2 months), middle-aged (12 months) and aged (24 months). Feces were weighed, and melatonin was extracted and measured by ELISA (IBL International, Hamburg, Germany). A very high level of melatonin was observed in the stools of young mice, such that levels increased at the light to dark transition (Fig. 4.3). These levels declined in middle-aged mice and were lost altogether in the aged mice.

4.5 Aging, Circadian Rhythms, and the Gastrointestinal Microbiome

It has recently become very clear that the microbiota that reside within our gastrointestinal system is critically important for our health (Cho and Blaser 2012). These microorganisms may comprise some 1000 species of bacteria, protists, fungi and other commensal, symbiotic and parasitic organisms whose mutual interactions and community form our "microbiome", an ecosystem within us that is important for the biosynthesis of vitamins such as vitamins B and K, interacts with our immune systems and affects the efficiency of digestion (O'Toole and Jeffery 2015; Bischoff 2016).

As with gastrointestinal function itself, aging affects the numbers and complement of microorganisms residing in the tract, and, as with the system itself, the microbiome is affected during aging by a wide array of factors. In human feces from volunteers ranging from 20 to 82 years of age, Anand et al. (2017) have reported a reduction in the abundance of members of the Phyla Actinobacteria and the family Bifidobacteriaceae in aged donors (age $\geq$ 60) relative to younger donors. However, the total bacterial diversity was not significantly altered. Mariat et al. (2009) found by quantitative PCR of a select group of enteric bacteria that the ratio of Firmicutes to Bacteroides to decreases with age. In laboratory mice, age-associated changes in the gut microbiome are associated with an increase in the pro-inflammatory marker MCP-1 (Conley et al. 2016). There is a decrease in Clostridiaceae and Deferribacteriaceae in aged mice compared to young C57Bl/6 mice.

To complicate the impact of aging on the human microbiome, the increase in drug usage as people age affects the microbiome as well. The use of NSAIDS alters the gut microbiome in drug-specific fashion, although use of NSAIDS in general differentiates drug users from non-users (Rogers and Aronoff 2016). For example, NSAIDS specifically increase the complement of Enterobacteriaceae and Bacteroidaceae, and the specific components of the microbiome were differentially affected by combinations of drugs commonly used by elderly patients.

The host circadian clock also affects the composition and luminal location of the microbiota. While total microbial activity may not vary over the time of day, individual taxa are rhythmic with different phases over the course of the day (Mukherji et al. 2013; Liang et al. 2014; Thaiss et al. 2014). For example, the relative abundance of fecal *Lactobacillus reuteri* in mice peaks at ZT 12 (Lights out), while the relative abundance of *Dehalobacterium* spp. peaks at ZT 24/0 (Lights on), depending largely on differing metabolic signatures (Kyoto encyclopedia of genes and genomes; KEGG; Thaiss et al. 2014). Disruption of the host's circadian clock by *per1/per2* double knockout (Thaiss et al. 2014), by *Bmal1* knockout (Liang et al. 2015) or by *Clock* mutation (Voigt et al. 2016) alters fecal bacterial composition. In addition to daily changes in microbial composition, rhythms of the biogeography of motile bacteria within the gastrointestinal tract such that bacteria associate with the intestinal epithelia of mice during the night (Thaiss et al. 2016). Simulated jetlag also disrupts daily patterns of KEGG pathways such as vitamin metabolism, nucleotide metabolism and two component/secretion systems (Thaiss et al. 2014) and shifts the microbial community structure by increasing gut Firmicutes bacteria and decreasing Bacteroides (Voigt et al. 2014).

We were curious whether the microbiota within the lumen of the gastrointestinal tract were sensitive to the melatonin we (Fig. 4.3) and others (Bubenik 1980; Huether 1994; Bubenik and Konturek 2011) have shown within the lumen. We therefore queried metagenomics data from GenBank and the Human Microbiome Project for bacterial sequences that resembled the human melatonin receptors. These analyses revealed several sequences in enteric bacteria that were 24–42% identical to the sequences of the MT1 and MT2 melatonin receptors (Paulose and Cassone 2016; Paulose et al. 2016). Among these was the magnesium transporter (MntH) of the proteobacterium *Enterobacter aerogenes.* After attempting several

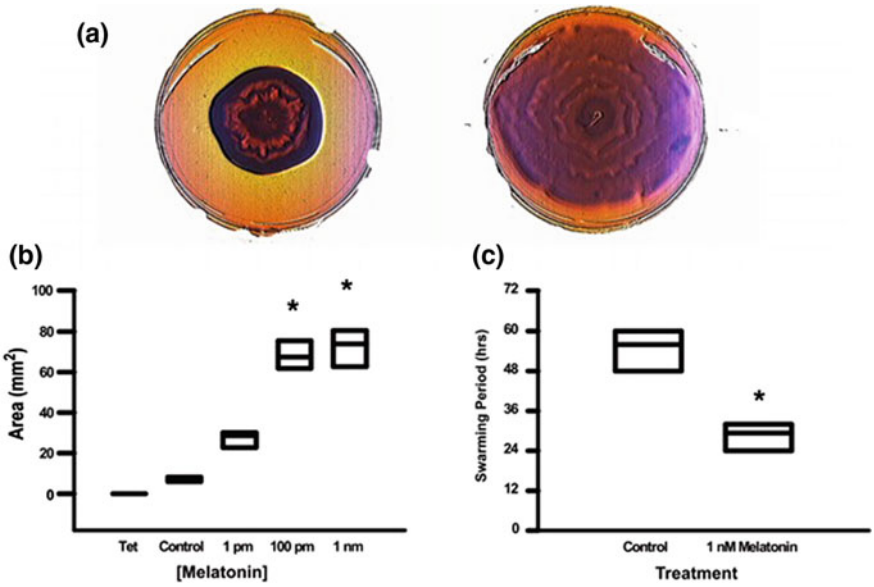
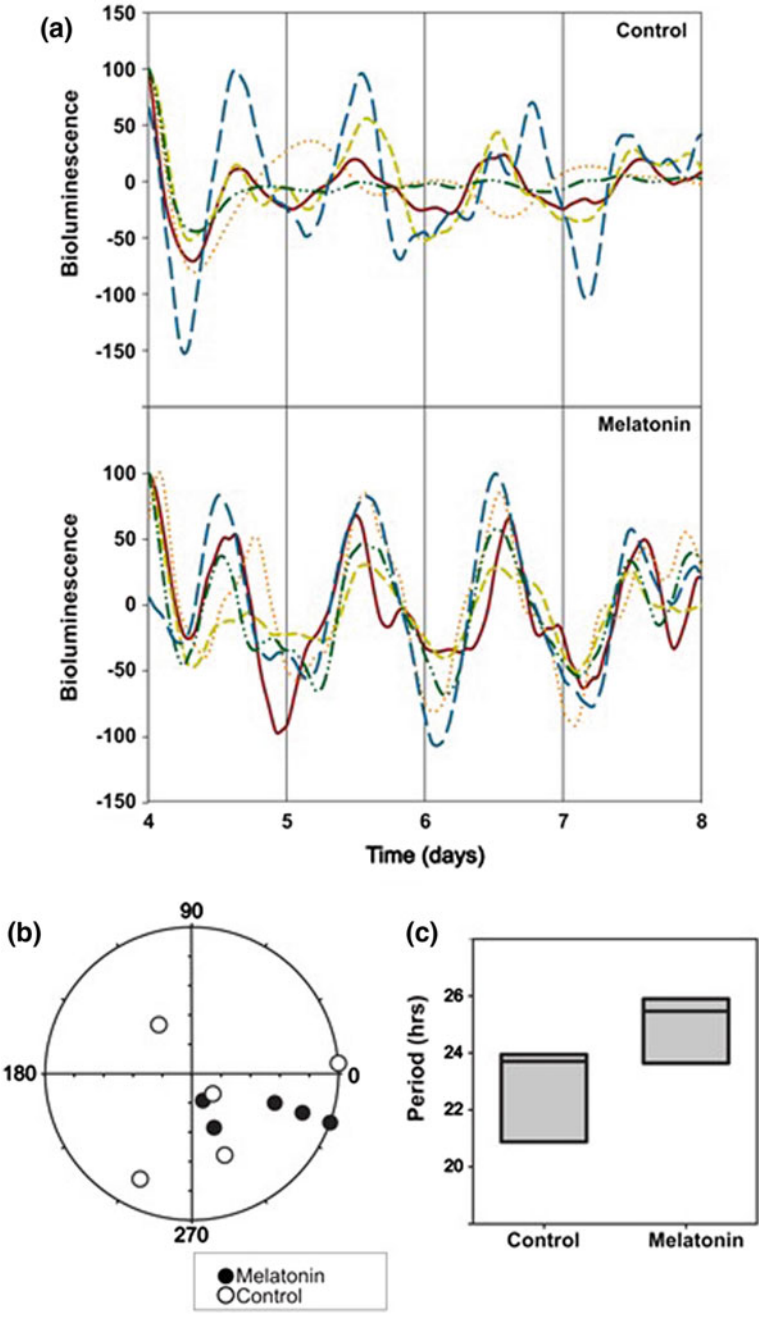


Fig. 4.4 Swarming behavior in *E. aerogenes* is induced by melatonin and occurs with a circadian frequency. **a** Swarming behavior in control treated (*left*) cultures versus treatment with 1 nM melatonin (*right*). Images were equally enhanced using "Bump Map" in GIMP software to highlight banding patterns. **b** The increase in swarming was only seen at 100 pM and 1 nM concentrations of melatonin. **c** The period of swarming behavior was approximately 50 h in the absence of melatonin but coalesced to 25 h in the presence of 1 nM melatonin. * = p value < 0.001 compared to vehicle treated cultures, n = 16 cultures per treatment (modified from Paulose et al. 2016 with permission)

different assays with disappointing results, we found that low concentrations of melatonin ranging from 1 pM to 1 nM increased swarming behavior in *E. aerogenes* on semi-solid agar (Fig. 4.4). This effect was dose-dependent and specific to melatonin, since other indolic compounds were ineffective. It was also specific to *E. aerogenes,* since neither *Escherichia coli* nor *Klebsiella pneumoniae* exhibited this behavior in culture. We were surprised to note that the increased swarming revealed daily banding in the cultures (Fig. 4.4). We therefore transformed the bacteria to express luciferase from *Aliivibrio fischeri (luxCDABE)* fused to the promoter for the flagellar stator *MotA (MotA::luxCDABE)*, such that bioluminescence is induced when the bacteria become motile. Interestingly, bioluminescence, measured in either an Actimetrics Lumicycle or Perkin Elmer IVIS imaging system, emanating from these bacteria was rhythmic with a circadian period of approximately 25 h (Fig. 4.5). The presence of melatonin in the media had no effect on this circadian period, but did synchronize the cultures. Importantly, the periods of these oscillations were invariant from 27 to 40 °C, exhibiting a Q_{10} of 0.96 and demonstrating that this bacterial clock was temperature compensated, considered an exemplar property of all circadian clocks. Yet, when placed in cycles of high (37 °C) and low


(a)
Control
Melatonin
Bioluminescence
Bioluminescence
150
100
50
0
-50
-100
-150
4
5
6
7
8
Time (days)
(b)
90
180
0
270
Melatonin
Control
(c)
Period (hrs)
26
24
22
20
Control
Melatonin

◂**Fig. 4.5** Bioluminescence recording of MotA::luxCDABE transformed *E. aerogenes* exhibits a self-sustained circadian rhythm at 34 °C. **a** Bioluminescence tracings from 5 control cultures and 5 cultures in 100 pM melatonin. **b** Phases of control rhythms are random, while melatonin treated cultures are synchronized. **c** Circadian period is not affected by melatonin (modified from Paulose et al. 2016 with permission)

(36 °C) temperatures (HL 12:12), these cultures entrained with a stable phase relationship to the HL cycle (Paulose et al. 2017). We have since shown that MntH::lucCDABE is also rhythmically expressed in this bacterium (Paulose et al. 2017). Thus, at least one bacterial component of the enteric microbiome possesses a temperature-compensated circadian clock that is synchronized by the host's gut hormone melatonin and entrained by natural changes on T_B. It is not known whether more species of bacteria possess clocks and entrain to their host, although it is likely. It is also not known whether other components of the microbiota merely react to dynamic, circadian changes in the host. Thirdly, it is not known whether bacteria that do not possess their own clocks synchronize to rhythmic bacteria via quorum sensing or other communication processes. Finally, it is not known how the process of aging affects these circadian interactions.

4.6 Conclusion and Prospectus

In toto, the scenario looks a little like this (with the caveat that there are major holes in our knowledge about these processes) (Fig. 4.6). Nearly all tissues express circadian patterns of a rhythmic gene network in which positive elements CLOCK and BMAL1 dimerize and stimulate the transcription of a large array of clock-controlled genes (Fig. 4.1). These clock controlled genes are diverse in their functions, depending on the tissues expressing them. In the brain, for example, the CLOCK/BMAL1 dimer stimulates the E-box in the promoter for hypothalamic AVP biosynthesis, and in the liver and elsewhere, it stimulates the E-box in the promoter of the Albumin-d-binding protein (Dbp). Among the genes stimulated in all tissues are the negative elements *per1, per2, per3, cry1* and *cry2*, which are transcribed and translated in the cytoplasm, where they oligomerized to reenter the nucleus and inhibit CLOCK/BMAL1 activation, closing a 24 h loop.

These rhythms are synchronized/entrained to the LD cycle by afferents in the eye forming a retinohypothalamic tract to oscillators in the SCN. The SCN in turn orchestrates downstream rhythms by entrainment of peripheral oscillators within the brain and in peripheral organs through diffusible factors in the brain and neural afferents throughout the body. In parallel, the timing of a meal can entrain behavior and the physiology of peripheral organs via mechanisms that are at this point unknown but that are at least in part independent of SCN and clock gene activity. Among the peripheral organs that express a circadian clock, the gastrointestinal system is capable of circadian rhythms of gene expression and physiology, which are dependent upon the expression of clock genes, which can be synchronized to

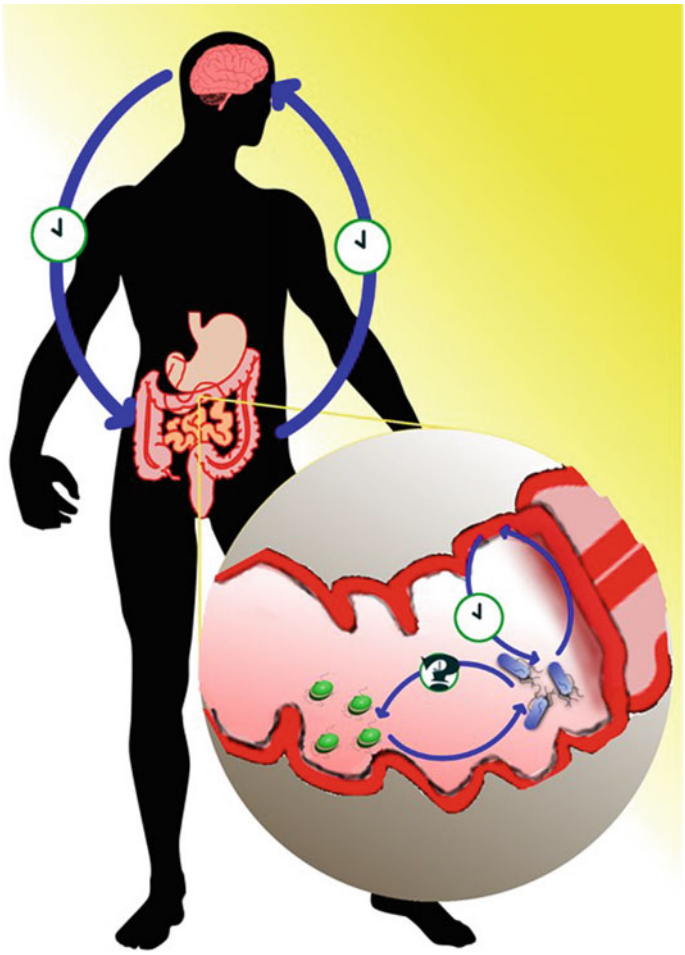


Fig. 4.6 Circadian rhythms are generated in both the suprachiasmatic nucleus (SCN) of the brain as well as in the gut. These rhythms are independent of, but feed back onto, each other. Within the gut, previous studies have shown that bacterial signaling to the gut via TLRs may influence circadian rhythms. The discovery of an enteric bacterium that expresses circadian rhythms and is sensitive to melatonin adds a novel signaling modality, and suggests that the rhythmic gut environment acts as a *zeitgeber,* or time-giver, to the microbiome. Whether or not this signal is propagated to other, insensitive bacteria, remains to be determined (from Paulose and Cassone 2016 with permission)

the LD cycle by SCN efferents through the sympathetic nervous system and which can also be directly synchronized by a timed meal. The gastrointestinal clock in turn orchestrates daily and circadian patterns of microbiotic diversity, physiology and gene expression. The mechanisms by which the microbiome's rhythms operate are unknown. However, new research in our lab indicates at least one species of enteric

bacterium, *E. aerogenes,* expresses its own circadian clock that is entrained by daily patterns of T_B and the gut hormone melatonin.

Each level of this hierarchical system, from the SCN to the peripheral tissues to the microbiome are subject to the onslaughts incumbent with age. These may be due to the natural program of aging, but they are complicated by many factors, including increases in inflammation, disease, changes in diet, increases in the use of pharmacological agents and many other factors. Further, there are many holes in our knowledge worth exploring. What are the mechanisms by which food entrains circadian clocks if they are independent of canonical clock genes? If the central clock gene rhythms within the gastrointestinal system are unaffected by age, what are the mechanisms by which rhythms of gastrointestinal output diminish over time? How is the microbiome in toto regulated by the circadian clock of the host? If only a few species of microbiota express clocks, how do non-rhythmic species synchronize rhythms? And how do these dynamics change during aging? Perhaps, most interestingly, how do circadian changes in our microbiome affect the host's physiology and behavior?

As can be seen, we have learned quite a bit about circadian organization and how it regulates gastrointestinal function. However, we have raised new questions about the mechanisms of aging. We have even posited new questions we never thought to ask.

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Chapter 5
Circadian System and Aging in Rodent Models

Andrey V. Panchenko, Ekaterina A. Gubareva and Vladimir N. Anisimov

Abstract Biological clock, or circadian rhythms (CRs) are an indispensable feature of nearly all living organisms; they may be observed at behavioral, physiological or cellular level. However, in mammals internal clock becomes less rhythmic and more vulnerable to external disturbing stimuli with age. In this chapter we briefly describe rodent circadian system physiology, rhythms and clock genes disturbance in aging and age-related diseases, models of genetic (clock genes mutant/knockout mice) and environmental (light-dark regimens manipulation) circadian disturbance. In the end some interventions which may be used to improve circadian output are considered.

Keywords Circadian rhythms · Aging · Animal model · Clock genes
Age-related diseases

5.1 Introduction

CRs have appeared in evolutionary timescale as early as in unicellular organisms. Their first function was to protect DNA from injury by UV-radiation through shifting its synthesis to night hours. In mammals, a relatively small number of cells proliferate, and yet fewer are subjected to solar radiation, though metabolism, free radicals scavenging and DNA synthesis are still regulated by cellular clock. Circadian rhythmicity in animals helps to anticipate environmental changes as well as to occupy an ecological time niche being active in particular during daytime.

In mammals circadian timekeeping system is highly conserved at an anatomical and genetic level. It's organized in hierarchical manner and consists of the central pacemaker capable to synchronize its rhythm with external light cues and transmit this information to peripheral oscillators. At the cellular level circadian clock consists of clock genes and their protein products which make up several

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transcriptional-translational feedback loops. Internal cycle is about 24 h long but it is always adjusted by the signals from central pacemaker. Melatonin is considered the main humoral time-keeping factor.

Aging circadian system fails to orchestrate peripheral rhythms precisely, so rhythmic function like rest-activity cycles, hormonal synthesis and antioxidant activity CR are disturbed.

Age-related decline in circadian function can significantly worsen life quality not only because of sleep loss or fragmentation. Cognitive, cardiovascular and metabolic disorders are also related with circadian system, so chronodisruption may seriously aggravate these pathologies. Animal models have revealed that both aging affects CR and CR disruption (environmental or genetic) leads to premature aging and age-related disorders manifestation.

5.2 Mammalian Circadian System

Nearly all physiological processes in mammalian organism undergo diurnal variations. To match a "circadian rhythm" definition, it should (a) have an endogenous period around 24 h; (b) have an ability to entrain in response to external stimuli; (c) exhibit temperature compensation. A list of rhythmic functions includes locomotor activity, body temperature, heart rate and blood pressure, synthesis and secretion of nearly all hormones, various metabolic processes, gene expression, etc. (Bell-Pedersen et al. 2005). However, only some of them may be measured with non-invasive methods (locomotion, sleep-wake cycles, body temperature, feeding and drinking rhythms), and most of CRs research is focused on these functions (Nakamura et al. 2008). The most evident rhythm is behavioral one, meaning rest and activity cycles, may be simply observed and assessed with use of actograms, special plots where daily activity patterns are aligned with another. With such representation, phase shifts may be seen (Jud et al. 2005). List of protocols to evaluate CRs consolidation are used: constant conditions (LL or DD), phase shift or jet-lag, long or short light cycles and complete chronodisruption.

5.3 Central and Peripheral Oscillators

Any clock, not only biological, has an internal mechanism generating rhythmic ticking, an input mechanism to synchronize clock with astronomical time and output signals need to transmit information. Suprachiasmatic nuclei (SCN) role as a circadian pacemaker was discovered in early 1970s, but exact mechanism of light sensitivity was disclosed only in 2002 with detection of special cells in retina percepting non-visual photic information. When neuronal projections from retina to SCN (retinohypothalamic tract, RHT) were revealed, its role in generating CR and entrainment to external light regimen became clear (Honma et al. 2012).

In mammals clockwork is located in SCN nuclei—each consisting of about 10,000 cells just above optical chiasm. Destruction of this brain region made animals behavior completely arrhythmic, and SCN transplantation restored rhythmicity (Li and Satinoff 1998). As SCN transplantation at least partially restores CR, even when it is transplanted in capsule which does not allow axons outgrowth, existence of some humoral factors transmitting rhythmic signal has been postulated (Silver et al. 1996). These factors include vasopressin, prokineticin 2 (PK2) and cardiotrophin-like cytokine (CLC). Corresponding genes contain E-box in their promoter areas and thus are regulated by clock genes in circadian manner. Their rhythmic release regulates CRs of body temperature, hormones synthesis and rest-activity cycles (Li et al. 2012).

Every single neuron in SCN is able to generate 24 h rhythm, but the rhythm strength depends on synchronization, with light being the most potent entrainment cue. Neurons in SCN coordinate their activity through paracrine neuropeptide signals as interfering synaptic vesicles formation results in disordered neuron firing. It was shown that vasoactive intestinal peptide (VIP), arginine-vasopressin peptide (AVP) and gastrin-releasing peptide (GRP) are responsible for synchronization in SCN (Lee et al. 2013; Hastings et al. 2014). Disruption of this coupling results in loss of rhythmic SCN output as VIP or VPAC2 (VIP receptor) knockout mice exhibit arrhythmic phenotype (Hastings et al. 2014).

While light is on, SCN neurons send inhibiting stimuli to pinealocytes, while in darkness this turns off, releasing melatonin synthesis by pinealocytes. Melatonin is much more ancient than pineal gland as it is found in unicellular eukaryotes. This hormone is synthesized from tryptophan mainly at night in diurnal and nocturnal species. It has two membrane G-protein coupled receptors MT1 and MT2 (Dubocovich et al. 2010). Melatonin was also shown to influence nuclear receptors (RORs and Rev-ERBs) activity and thereby regulate clock genes expression (Hazlerigg et al. 1996). In addition to axons regulating pineal gland function, SCN has neuronal projections to hypothalamus and the midline thalamus. Multiple peripheral oscillators exist in a mammalian organism though their phases may not coincide. Each tissue possesses some mechanisms to synchronize its functional units through paracrine and cell-to cell signaling. Besides, each cell has its own molecular clock. Here we'll regard liver as an example of a peripheral oscillator. This tissue is highly active metabolically, and its functioning is restricted to time of feeding to uptake nutrients and remove toxic compounds. Usually feeding coincides with activity phase but sometimes food uptake rhythm may be disrupted. This accounts especially for humans with irregular regimen or nighttime eating syndrome (NES). In laboratory rodents this situation is modelled with the restricted feeding protocol when chow is given only 1–2 h per day. It was shown that in such conditions mouse liver re-entrains its molecular clock according to feeding time while SCN rhythm is still entrained by light (Mendoza et al. 2005). Central clock does not strictly regulate peripheral ones. Liver-specific Rev-Erba knockout mice, which retained SCN rhythm, have overlapping, but not similar liver circadian transcriptome compared with WT mice (Vieira et al. 2014). Thus peripheral tissues have their own rhythmicity while SCN orchestrates peripheral tissues.

5.4 Circadian System in Aged Rodents

5.4.1 Age-Related Circadian Rhythm Disturbance

Most rhythmic functions of the body as well as synchronization of peripheral clocks are disturbed with aging. Some common features of ageing rhythms have been found in people and animals. Older rodents exhibit reduced rhythmicity in body temperature, sleep-wake cycle, and locomotor activity (Turek et al. 2001; Duncan et al. 2012). However, animals used for aging research may exhibit minimal changes. Some old and healthy survivors may have more consolidated rhythm than animals that have died earlier (Turek et al. 2001).

Amplitude decrease and **fragmentation** of daily activity rhythm is observed in mice, rats and hamsters. In old animals time of activity is shorter and it is disrupted into short intervals (Nakamura et al. 2011; Valentinuzzi et al. 1997; Turek et al. 2001). Decrease in amplitude was shown also for body temperature and corticosterone rhythm. Interestingly, interindividual difference in rhythm disruption was observed in rats and mice: while most animals in old age had dampened amplitude of rest-activity cycles, others maintained pronounced CR. It should be noted that different oscillators undergo aging and rhythm degradation with different speed. For example, corticosterone rhythm was dampened earlier than activity rhythm, while body temperature rhythm was still pronounced in animals with decreased activity amplitude (Weinert 2000).

Phase shift is observed in old rodents. In most investigations phase advanced behavior (earlier rest and activity onset) in mice and hamsters were observed, though in C57BL mice phase delay was shown (Valentinuzzi et al. 1997).

Free-running period shortening has been observed in some species and/or strains (Witting et al. 1994) which may contribute to phase advanced behavior.

Entrainment is impaired in aged mice and hamsters (Sellix et al. 2012; Scarbrough et al. 1997). As animals need more circadian cycles to resynchronize, they become more sensitive to jet-lag (Davidson et al. 2006).

It's important to understand what components of the clock wear out during lifetime. Circadian system loses sensitivity to entraining light cues with aging though lens transmission in old golden hamsters does not change significantly (Zhang et al. 1998). Using mice that lack rod and cone photoreceptors Semo et al. (2003) has shown 40% reduction in retinal ganglion cells in aged animals. This reduction may contribute to lower circadian photosensitivity found in older animals and people. Central clock's ticking also becomes less rhythmic with age.

5.4.2 Aging Suprachiasmatic Nucleus

Aging SCN does not lose many neurons nor do they undergo atrophic changes though SCN functions decline (Farajnia et al. 2014; Duffy et al. 2015).

Recording SCN multiunit neural activity in vivo in mice has revealed reduction in neuronal firing rhythm amplitude. Moreover, CR was degraded in the subparaventricular zone, one of the main outputs of the SCN (Nakamura et al. 2011). Electrophysiological measurements of neurons in brain slices containing SCN from old rats, mice and hamsters have shown changes in neuronal firing occurring with age (Satinoff et al. 1993; Watanabe et al. 1995; Aujard et al. 2001). There's evidence that neuronal activity in SCN of old mice is desynchronized (Farajnia et al. 2014). Furthermore, the number of cells expressing VIP and AVP, peptides required for orchestrated neuron work, diminishes in aged rats' SCN (Roozendaal et al. 1987). SIRT1 expression declines in old mice brain, besides, deficiency in this gene results in age-resembling rhythm disturbance. SIRT1 overexpression protects mice from age-related decline in rhythmic functions and clock genes (CGs) expression (Chang and Guarente 2013). Transplantation of fetal SCN into third ventricle of old rats has been shown to improve their circadian rhythmicity (Li and Satinoff 1998). Moreover, wild type SCN transplantation into arrhythmic Cry1/Cry2 knockout or Clock mutant mice generated behavioral rhythms in absence of any functional peripheral oscillators (Sujino et al. 2003). Thus, rhythmic SCN output signals are indispensable for CR maintenance throughout lifetime.

5.4.3 Aging Pineal Gland

Pineal melatonin synthesis decreases and has blunted CR amplitude in aged animals and people. In rodents pineal gland undergoes mild morphological changes with age. In rats some authors haven't described any significant age-related changes while others have reported decrease in the total number of pinealocytes, increased thickness of the connective tissue capsule and the relative amount of connective tissue cells, calcified concretions, and some ultrastructural cellular changes (Humbert et al. 1997). However, decreased innervation by sympathetic axons was demonstrated in the aged rat pineal gland (Kuchel et al. 1999; Schmidt et al. 2006). Decrease in pineal melatonin production rhythm seems to be linked to loss of axonal projections from SCN bringing information on light-dark cycle. Morphological changes reflecting reduced synthetic and secretory activity may be a consequence of decreased innervation. Additionally, melatonin signal is thought to play an important role in pinealocytes maintenance. When melatonin-deficient C57BL/6J mice were compared with normal CBA strain at 10 month age, the latter had more pinealocytes though in young age pineal glands of both strains they were similar (Cernuda-Cernuda et al. 2000). In general, circadian system aging manifests in desynchronization of rhythms at behavioral, tissue and cellular level possibly due to decrease of melatonin level.

5.4.4 Molecular Clock

Changes in SCN function may occur due to loss of coordinated clock genes expression. There are numerous studies that reveal changes in clock genes expression in SCN, other parts of the brain and peripheral tissues in aged animals (data is summarized in Table 5.1). Age-related disturbance of clock genes is equivocal and may be masked by light-dark cycles which entrain their expression in SCN. SCN neurons had lower Per2 expression amplitude and longer circadian period in old mice kept in constant darkness than in mice of the same age kept in normal LD cycle. Moreover, neurons in DD-housed mice were not synchronised (Nakamura et al. 2015).

Aging of circadian system is a complex process comprising multiple changes at behavioral, anatomical and cellular level with both central and peripheral oscillators involved. Though a large set of data has been obtained for more than 50 years of research on circadian system, a single cogwheel broken in the aged clock has not been found.

Aging leads to circadian disruption—is the link bidirectional? In further sections we will discuss effects of circadian disruption, both environmental and genetic, on age-related changes and interventions which influence circadian system and aging.

5.5 Circadian Rhythms in Rodent Models of Accelerated Aging and Age-Related Pathologies

5.5.1 Mice with Accelerated Aging

Mice prone to accelerated senescence (SAMP) have been established by Takeda and colleagues as a result of AKR/J mice inbreeding (Takeda 1999). Several SAMP mouse substrains having lifespan about 10 months and demonstrating early age-related changes have been obtained. SAMP8 and SAMP10 mice were shown to have impaired CR, SAMP8 in addition exhibit Alzheimer disease-like phenotype relatively early, including neuron loss and marked oxidative stress (Pang et al. 2004; Morley et al. 2012). Seemingly age-related changes, especially in the brain, affect circadian rhythmicity.

5.5.2 Clock Genes in Rodent Models of Age-Associated Diseases

Neurodegenerative diseases. Age associated neurodegenerative diseases including Parkinson's disease (PD), Alzheimer's disease, and Huntington's disease share such symptoms as progressive death of neurons, cognitive or motor disorders and

Table 5.1 Age-related changes of CGs expression in different rodent species

Species	Tissue	Changes in CGs expression or clock protein level	Other effects	Authors
Syrian hamster	SCN	Changes in Clock and Bmal1 rhythmicity	Per1 light induction ↓	Kolker et al. (2003)
Syrian hamster	Skeletal muscle	No changes in Per2 and Bmal1 rhythm	ND	Duncan et al. (2013)
	SCN, cingulate gyrus, hippocampus, olfactory lobe	Loss of Bmal1 rhythm, Per2 and Bmal1 amplitude ↓	ND	
Mouse	Hippocampus, amygdala, nuclei of basal hypothalamus	Changes in amplitude and/or phase of Clock and Bmal1 protein synthesis	Retained Clock and Bmal1 expression in SCN	Wyse and Coogan (2010)
Mouse	SCN	Loss of Bmal1 rhythm, rhythmic Cry1	ND	Bonaconsa et al. (2014)
	Liver	Per1, Per2, Cry1 amplitude ↓, Clock rhythmic expression	ND	
	Heart	Cry2 amplitude ↓, Clock rhythmic expression	ND	
Mouse	SCN	No changes in Per1, Cry1, Clock protein synthesis rate, Per2 ↓	Change the rhythm of activity in old animals	Weinert et al. (2001)
Rat	SCN	Loss of Cry1, Cry2 and Bmal1 rhythm, shift of Per1, Per2 phase	Partial restoration of behavioral and clock Genes rhythmicity with melatonin	Mattam and Jagota (2014)
Rat	SCN, PVN, pineal gland	No changes in Per1, Per2, Cry1 rhythm	Per1, Per2 light induction ↓	Asai et al. (2001)
Rat	SCN, retina, pituitary gland	No changes in Per1 rhythm	Per1 period ↓ in SCN culture from old animals	Yamazaki et al. (2002)
	PVN, pineal gland, kidney,	Shift of Per1 phase		
Rat	SCN	Loss of Bmal1, Cry1, and Cry2 expression rhythm	Restoring CR of CGs with melatonin	Sandu et al. (2015)
Rat	Skin culture	Per1 amplitude ↓	ND	Sandu et al. (2015)
	Dermal fibroblasts	Per1 period ↓	ND	
Rat	Liver	Per2 ↓ in the evening	ND	Claustrat et al. (2005a, b)
	Heart	Trend to Per2 expression reduction, Bmal1 ↑	ND	

↓ Decrease, ↑ Increase, *ND* No data

disruption of activity, sleep-wake cycle, and body temperature CRs. Aberrations of different rhythmic functions are also observed in rodent models of these diseases.

Degeneration in dopaminergic neurons, which occurs in Parkinson's disease patients and causes characteristic tremor, may be induced in rodents by some chemicals (6-hydroxydopamine, 1-methyl-4- phenyl-1,2,3,6-tetrahydropyridine, rotenone) or it occurs spontaneously with age in genetically modified mice (alpha-synuclein-overexpressing and MITOPARK mice). All the mouse and rat models demonstrate blunted rest-activity and CGs expression rhythms, as well as other rhythmic functions disturbance (Videnovic and Willis 2016). However, administration of levodopa (Boulamery et al. 2010), melatonin (Mattam and Jagota 2015) or ATP (Hayashi et al. 2013) has been shown to produce a neuroprotective effect and partially restores rhythmic functions.

Alzheimer's disease in people is characterized by the accumulation of protein aggregates consisting of τ-protein and β-amyloid in the brain tissue, memory disorders, sleep disturbance and changes in the CR of body temperature and activity, as well as the loss of rhythmicity in melatonin synthesis (Harper et al. 2005). The transplantation of cells with the β-amyloid overexpression into the SCN of rats changed their activity and body temperature rhythmicity a few days after the treatment (Tate et al. 1992). β-amyloid has been shown to increase Bmal1 protein degradation (Musiek and Holtzman 2016). It's supposed that synchronization between the master and peripheral oscillators is disturbed in Alzheimer's disease as similar changes are found in CLOCK gene expression in pineal gland in patients with Alzheimer's disease and in rats with destructed SCN (Wu et al. 2006).

Huntington's disease is an autosomal-dominant illness that emerges in individuals carrying the huntingtin allele with a large number (over 36) of CAG repeats. Its symptoms include hyperkinesia, dystonia, cognitive and mental disorders, sleep-wake rhythm disturbance, as well as neuronal degeneration in striatum and the large hemispheres of the hypothalamic area (Kassubek et al. 2004). Mouse models of Huntington's disease include *R6/2* lines with 140 or 250 CAG repeats in Htt gene and transgenic BACHD knock-in mice with artificial bacterial chromosome carrying human Htt with multiple repeats. Both models exhibit rest-activity and body temperature CR disturbance even prior to neurological symptoms progression (Musiek and Holtzman 2016). CR disturbance and CGs expression in mouse models and in patients with neurodegenerative diseases seem to be a secondary disorder related to neuronal network damage. For example, normal CG expression has been observed when cultivating SCN tissue from the *R6/2* mice, as compared with the SCN of the same mice in vivo (Musiek and Holtzman 2016).

Metabolic disorders. Studies in animals have shown that the circadian disruption in people and laboratory rodents leads to metabolic disorders and obesity (Wang et al. 2014; Vinogradova et al. 2009; Lucassen et al. 2016). To maintain metabolic equilibrium, the expression rhythms of the genes participating in metabolism should coincide with the rhythms of food consumption. CGs expression in β-cells, levels of hormones controlling the nutritional behavior and glucose metabolism, as well as mRNA of key enzymes determining the rates of glycolysis, oxidative phosphorylation, and the oxidation of fatty acids, oscillate over the

circadian cycle (Vieira et al. 2014; Marcheva et al. 2010; Panda et al. 2002). However, metabolic disorders influence CR and CGs expression in many rodent models of genetically or chemically induced diabetes (Table 5.2). As will be described further, CGs mutations negatively impact circadian metabolic organization; some of CGs knockout/mutant mice are prone to metabolic syndrome.

5.5.3 Rodent Models of Circadian Disruption

Circadian rhythm disruption occurs when rest-activity cycles and other internal rhythms are not adjusted to environmental light-dark regimens and not synchronized among each other. Rhythm disturbance is normally associated with aging, but in people it is common in adults because of artificial light pollution and social factors like shift work, travelling across time zones and using electronic devices and light at night. Irregular day and night length is also a factor compromising circadian system (Anisimov et al. 2012). A lot of studies on shift and night work have revealed its adverse effects on human health, namely sleep disruption, mood disorders, metabolic impairment and obesity, cardiovascular anomalies, gastrointestinal problems and increased cancer incidence (Touitou et al. 2017). Though investigation of CR and aging in people is methodologically limited, animal experiments involving circadian disruption provide a link between CR disruption and accelerated aging through evaluation of age-related markers and longevity. Interestingly, rats subjected to chronic jet-lag protocol similar to rotating-shift nurses' schedule demonstrated a similar reduction in activity CR amplitude in spite of natural 12 h difference in activity peak (Rea et al. 2008). In laboratory rodents several protocols of circadian clock disturbance are used, including light mode manipulation, surgical intervention and molecular clock mutations/knockout models.

5.5.4 Light Regimen

Experimental conditions used are either constant (LL or DD) or imply irregular light cycles like chronic jet lag or northern natural light. DD or blindness makes rhythm free-running although internal synchrony may be preserved because of intact melatonin and SCN output signals. Constant light, light-dark cycle reversal or pinealectomy dramatically decrease melatonin synthesis. Few weeks in LL are enough to abolish or dramatically delay prolactin and corticosterone rhythms in rats (Claustrat et al. 2008). Clock genes expression in SCN also changes in LL conditions (Sudo et al. 2003). In aged mice SCN becomes more vulnerable to LL desynchronizing effect due to weakening of neuronal circuits (Polidarová et al. 2017). Chronic circadian desynchronization induced by light conditions or surgery decreases mean life span in rodents, impairs glucose metabolism, induces metabolic

Table 5.2 Clock genes in rodent models of diabetes mellitus

Diabetes type or experimental model	Studied tissue	Changes in the level of clock-gene expression in diabetes	Serum glucose	Serum insulin	Body weight	Other effects	Authors[a]
Rats, streptozotocin	Heart	Phase shift of Bmal1, Clock, Cry1, 2, Per1, 2, 3	↑	ND	ND	Change in the rhythm of blood glucose fluctuations	Young et al. (2002)
Mice, streptozotocin	GIT	Phase shift of Per2, 3	↑	ND	ND	No changes in the rhythm of feed consumption were observed	Bostwick et al. (2010)
	Lung	=					
	Kidney	=					
Neonatal administration of streptozotocin to mice	Liver	Bmal1, Cry1, Per ↑	↑	↓	=	Disturbed rhythms in the expression of the genes controlling glucose metabolism	Yang et al. (2013)
kk-Ay diabetic mice	Liver	Disturbed rhythm of Per3, Bmal1, and Npas2	↑	↑	↑	Changes in the rhythm of expression of 13 genes in liver	Ando et al. (2006)
Goto-kakizaki rats	Liver, fatty tissue	No difference in the rhythm of the Clock, Bmal1, and Cry1	↑	ND	=	–	Ando et al. (2009)
Diabetic Zucker rats with obesity	SCN	=	↑	ND	↑	–	Motosugi et al. (2011)
	Liver	Cry1, Per1 ↓					
	Aorta	Per1 ↑					
	Heart	=					
	Fatty tissue	=					

(continued)

Table 5.2 (continued)

Diabetes type or experimental model	Studied tissue	Changes in the level of clock-gene expression in diabetes	Serum glucose	Serum insulin	Body weight	Other effects	Authors[a]
db/db diabetic mice	Arteries	Disturbed rhythms of the Per1, 2, Cry1, 2	↑	ND	↑	Disturbed contractility of vascular smooth muscles	Su et al. (2012)
	Kidney	Disturbed rhythm of Per1					
	SCN	Disturbed rhythm of Per1					

SCN The suprachiasmatic nucleus, *GIT* Gastrointestinal tract, ↑ Increase, ↓ Decrease, = No changes, *ND* No data
[a]Reviewed in Panchenko et al. (2017)

syndrome-like phenotype, influences immune response, reduces physical activity and performance, causes cognitive decline, increases spontaneous tumorigenesis, etc. (Tables 5.3 and 5.4; Anisimov et al. 2012; Belancio et al. 2014; Lucassen et al. 2016; Mizutani et al. 2016). However, light regimen effect on aging and associated carcinogenesis is highly dependent on genetic background of mouse strain (Table 5.4).

5.5.5 *Circadian Genes Mutations and Their Impact on CRs and Age-Related Disorders*

First circadian mutation, Period, was found in Drosophila. But in mice such mutant's circadian behavior isn't unlike its wild-type littermates'. In mammals clock genes are duplicated with each family comprising two or more members. These genes are functionally at least partially redundant except Bmal2, which can't be activated in Bmal1 absence. In case of other CGs, when one of the paralogs is absent, the other one may be activated. That's why Clock knockout mice in which this gene is completely absent express Npas2 in their tissues and exhibit virtually normal phenotype. At the same time mice with mutant Clock express non-functioning protein and exhibit loss of CR and other pathological phenotypic features. However, maintenance of both functional Per or Cry family members is important for intrinsic rhythmicity strength and entrainment range. Mutant mice have lower amplitude of behavioral rhythm, shorter period and can entrain better to short 16 h LD cycle (Erzberger et al. 2013). Interestingly, Cry1/Cry2 double-mutant mice demonstrate arrhythmic wheel-running behavior and lack of rhythmic clock gene expression in constant darkness whereas light partially restores activity rhythm (Albus et al. 2002).

CGs knockout mice reveal a link between circadian disruption, aging and age-related pathologies. For example, Clock and Bmal1 knockout mice have reduced lifespan and severe metabolic alterations. $Per2^{-/-}$ animals are prone to carcinogenesis, and in some models females have earlier onset of reproductive aging. Tissue-specific CGs knockout allows researchers to reveal the role of peripheral oscillators in the regulation of various physiological processes and exclude influence of the central clock. Moreover, models of tissue-specific rescue have been developed which allow to turn on a gene of interest in global knockout under specific promoter, so animals express a given gene only in tissues where the promoter is active. CGs tissue-specific knockout and tissue-specific rescue models are reviewed in detail by Birky and Bray (2014). Some CGs global and tissue-specific knockout phenotypes (with special attention to age-related disorders) are presented in Table 5.5.

However, in knockout mice non-circadian effects of CGs in prenatal and neonatal development may modulate their phenotypic features. In case of $Bmal^{-/-}$

Table 5.3 Summary evaluation of effects of various light/dark regimens on biomarkers of aging and homeostatic parameters in female and male rats

Parameters	Sex	Light/dark regimen		
		Natural lighting (NL)	Constant lighting (LL)	Light deprivation (DD)
Body weight	Male	↑	=	=
	Female	↓	↓	↓
Weight gain	M & F	↓	↓	↑
Progressive growth period	Male	↑	↓	↑
Stabile growth	Male	↓	↓	↓
Presenile period	Male	↑	↑	↑
Senile period onset	Male	↑	↑	↓
Food consumption	M & F	↑	↑	↓
Water consumption	Male	=	=	=
Maturity onset	M & F	↑	↑	↓
Estrous function switching-off	Female	↑	↑	↓
Diuresis	Male	↓	↓	↓
Morbidity	M & F	↑	↑	↓
Biochemical indicators in urine				
Glucose (age of appearance)	Male	↑	↑	↓
Ketones (age of appearance)	Male	↑	↑	↑
Behavioral, psycho-emotional and cognitive function, working capacity				
Locomotor activity	Male	↑	↑	↓
Psycho-emotional features	Male	↑	↑	↓
Cognitive function	Male	↓	↓	=
Dynamic endurance	Male	↓	↓	↑
Static endurance	Male	↓	↓	↓
Biochemical indicators in blood				
Glucose	M & F	↑	↑	=
Cholesterol	M & F	↑	↑	=
Beta-lipoproteins	M & F	↑	↑	=
Total protein	M & F	↓	↓	↑
Carbamide	M & F	↑	↑	=
Creatinine	M & F	↑	↑	=
The level of hormones in the blood				
Prolactin	M & F	↑	↑	↓
C-Peptide	M & F	↑	↑	=

(continued)

Table 5.3 (continued)

Parameters	Sex	Light/dark regimen		
		Natural lighting (NL)	Constant lighting (LL)	Light deprivation (DD)
Thyroid-stimulating hormone	M & F	↓	↓	=
Thyroxine	M & F	↑	↑	=
Triiodothyronine	M & F	↓	=	↑
Coefficient of homeostatic stability#	M & F	↓	↓	↑
Antioxidant system				
Liver	M & F	↓	=	=
Kidney	M & F	=	↓	=
Heart	M & F	↓	↓	=
Skeletal muscle	M & F	↓	↓	=
Life span	M & F	↓	↓	↑
Spontaneous carcinogenesis	M & F	↑	↑	↓

Reproduced with modifications from Anisimov et al. (2010), with permission
↑ Increase in effect; ↓ Decrease in effect, = No influence, # Ratio of total number of biochemical and endocrine parameters equal to relevant indices at the age of 3 months to total number of parameters studied (Vinogradova et al. 2009)

mice, inducible knockouts which express this gene until pubescence do not display lifespan shortening and arthropathies though with the gene turnoff their behavior becomes arrhythmic (Yang et al. 2016).

5.6 Interventions Improving Circadian Rhythms and Longevity in Rodents

Proper circadian system functioning is essential for health maintenance and longevity. Proximity of free-running period *tau* to 24 h is related to the lifespan in laboratory mouse strains (Wyse et al. 2010). As aging per se leads to decline in circadian output, the decline may be much more pronounced when circadian disruption or age-related diseases are imposed.

Light is the most important zeitgeber for both nocturnal and diurnal species. SCN neuronal activity has been shown to be tightly correlated with photic input (Nakamura et al. 2008). Bright light therapy is a promising approach to treatment of neurological diseases (Schroeder and Colwell 2013). However, circadian system may respond to other entraining cues. Below we summarize experimental data on environmental signals (light therapy) and different chronobiotics treatment.

Table 5.4 The effect of light/dark regimen on biomarkers of aging, longevity and development of spontaneous tumors in mice

Strain	Light regimen	Body weight of aged mice (g)	Fraction of mice with irregular estrous cycles (%)	Mean lifespan (days)	Aging rate (α)[c] ($days^{-3}$)	Number of tumor-bearing mice (%)	The age of the 1st tumor detection (mean latent period) (days)	Total number of tumors	Authors
129/Sv	LD	26.6 ± 0.3[a]	8.3[a]	763 ± 21.8	7.97 (6.74; 8.23)	86.7	500	39	Popovich et al. (2013)
	LL	31.6 ± 0.5[a]	68.4[a]	701 ± 20.7	9.39 (8.83; 10.9)	81.4	442	38	
HER-2/neu transgenic mice	LD	34.2 ± 1.26[b]	50.0[b]	281 ± 8.1	–	76.7	135 (236 ± 5.9)	75	Baturin et al. (2001)
	LL	32.4 ± 1.04[b]	26.7[b]	308 ± 10.8	–	76.0	188 (268 ± 4.7)	103	

LD Artificial standard light/dark regimen, *LL* Constant lighting

[a]At the age of 20 months

[b]At the age of 8 months

[c]Constant α in the Gompertz equation: $R = R_0 (\exp) \alpha t$, where R_0 = mortality at $t = 0$

Table 5.5 Age-related disorders in clock genes mutant mice

Genotype	Circadian-related phenotype	Other phenotypic features	Authors[a]
Bmal1 knockout	Disturbed rhythm of locomotor activity	Reduced lifespan, premature aging, increased sensitivity to oxidative stress, sarcopenia, arthropathy, osteoporosis, ablated glucose and triglycerides rhythm, altered glucose metabolism	Kondratov et al. (2006), Kennaway et al. (2013)
Clock knockout	Disturbed rhythms of feed consumption and locomotor activity, response to light is altered	ND	DeBruyne et al. (2007)
$Clock^{+/-}$	Longer period, reduced activity, altered Per1, 2 expression in SCN	ND	Kolker et al. (2004)
$Clock^{\Delta 19/\Delta 19}$	Disrupted behavioral and feeding rhythms	Metabolic syndrome, altered estrous cycles	Turek (2005)
$NPAS2^{-/-}$	Disrupted sleep-wake cycles	ND	Garcia et al. (2009)
$Per1^{-/-}$	Short behavioral activity period, arrhythmic adult SCN slices	Earlier reproductive aging in females,	Masubuchi et al. (2005)
$Per2^{-/-}$	Phase advanced locomotor activity rhythm, sleep disorders, altered feeding rhythm	Increased mortality, tumor incidence, higher body mass, loss of corticosterone rhythm	Fu et al. (2002), Yang et al. (2009)
$Per2^{-/-}$, $Per1^{-/-}$	Lack of CGs rhythmicity	Earlier reproductive aging in females	Bae et al. (2001)
$Per3^{-/-}$	Circadian period shortening	High-fat diet-induced weight gain in males	Dallmann and Weaver (2010)

(continued)

Table 5.5 (continued)

Genotype	Circadian-related phenotype	Other phenotypic features	Authors[a]
$Cry1^{-/-}$ $Cry2^{-/-}$	Loss of behavioral rhythms in DD, loss of Per genes expression and neuronal firing rhythm in the SCN	ND	Vitaterna et al. (1999)
B-cells-specific $Bmal1^{-/-}$	Normal rhythms of feed consumption and locomotor activity	Increased glucose and decreased insulin plasma level	Marcheva et al. (2010)
Cardiomyocyte-specific deletion or mutation of Bmal1, Clock, Per1 or Per2	Normal behavioral rhythm	Diverse signs of cardiovascular pathologies (cardiomyopathy, blood pressure alteration, myocardium metabolism, etc.), loss of myocardium rhythmic functions	Bonney et al. (2013), Bray et al. (2008), Lefta et al. (2012), Schroder et al. (2013), Tsai et al. (2010), Young et al. (2014)
Adipocyte-specific $Bmal1^{-/-}$	Disrupted feeding rhythm	Obesity under high-fat diet	Paschos et al. (2012)
Hepatocyte-specific $Bmal1^{-/-}$		Disrupted rhythm of genes involved in glucose metabolism	Lamia et al. (2008)

If not stated otherwise, rhythmic functions were assessed in normal LD cycle. *ND* No data
[a]Reviewed in Tsang et al. (2017), Panchenko et al. (2017), Birky and Bray (2014)

Physical activity and stress both affect hypothalamic–pituitary–adrenal and sympathetic–adrenal–medullary axes through shifting adrenal hormones synthesis (Mastorakos et al. 2005). Induced exercise changed rest-activity cycles in absence of other entraining stimuli in Syrian hamsters housed in DD (Reebs and Mrosovsky 1989). Timing of exercise or stressing stimuli matters: before the middle of subjective day phase advance is observed, while entraining stimuli in the end of activity phase have phase-delaying effect. Scheduled activity or stress in either the first or the second half of active phase under LD or DD conditions has been shown to shift differentially not only behavioral rhythms but also CGs expression (Schroeder and Colwell 2013; Tahara et al. 2017; Reebs and Mrosovsky 1989). Regular exercise may be helpful to reinforce CRs disturbed by aging or genetic background. In aged mice scheduled wheel running also improved behavioral rhythmicity compared to animals without wheel access (Leise et al. 2013). In mice with VIP or VIP receptor mutations which have disrupted CR, regular daily wheel

availability improved physiological and rest-activity rhythms (Power et al. 2010). In old people scheduled training also improves sleep timing and quality (Schroeder and Colwell 2013). Though exercise is a potent zeitgeber, molecular mechanisms of physical activity feedback to circadian system is still unknown.

Feeding. Evidently feeding occurs in active phase with gastrointestinal and metabolic functions anticipating it. When food consumption is temporally restricted, peripheral oscillators (liver, heart, kidney, pancreas, adipose tissue, and the gastrointestinal tract) rather than lighting schedule entrain feeding. SCN-lesioned mice entrain feeding cues faster than control ones perhaps due to absence of central pacemaker control (Potter et al. 2016). Scheduled feeding works as entraining stimulus in LL conditions when peripheral oscillators are desynchronized (Hamaguchi et al. 2015). Restricted scheduled feeding has been found to be a more effective zeitgeber, which manages to influence SCN phase, than normocaloric food (Mendoza et al. 2005).

Sleep. SCN has bidirectional neural connection with «sleep centers». Circadian system controls sleep, but it also receives information from other brain regions concerning sleep or awake state (Deboer et al. 2003). In mouse HD model pharmacological sleep imposition improved cognitive and motor functions and improved rhythmic Per2 expression (Pallier et al. 2007). Thus appropriate sleeping time or pharmacological induction of regular sleep may have beneficial effect on rhythmicity and life quality in elder individuals with neurodegenerative disorders.

Pharmacological interventions. Most cellular signaling pathways are interconnected, that's why many drugs hit multiple targets. It looks like molecular rule of six handshakes works in relation to CGs. Some of established pharmaceuticals can shift or reset molecular clock; such side effect may be beneficial in some situations like jet-lag and may be undesirable, e.g., in individuals with phase advance syndrome taking drugs that shorten circadian period. List of drugs and biologically active compounds influencing CRs includes caffeine, sildenafil, lithium, valproic acid, and serotonergic drugs (Schroeder and Colwell 2013; Tahara et al. 2017). However, all of them have specific established activity and some cause addiction.

It's beyond the scope of this chapter to describe all the drugs' mechanisms of action, so as an example metformin will be considered. Metformin is a biguanide widely used for diabetes treatment. Metformin activates AMPK, a sensor of glucose which phosphorylates CKI-epsilon, regulating Per2 degradation and circadian cycle length (Um et al. 2007). Chronic metformin treatment modulates CGs expression in different peripheral tissues (Barnea et al. 2012). Interestingly, metformin treatment mimics caloric-restriction which has been demonstrated to have beneficial effect on longevity and counteracts some age-related diseases (e.g. metabolic disorders and cancer) (Anisimov 2015).

Melatonin, an endogenous chronobiotic, has blunted peak of synthesis in old rodents and people; it is thought to contribute to age-related decline in rhythmic functions, besides, this hormone deficiency is involved in pathogenesis of many

age-related diseases. Therefore, exogenous melatonin may be administrated not only to improve sleep and rhythmicity but also to mitigate cardiovascular, metabolic and neurological disorders. In old rodents melatonin has been shown to improve phase shift entrainment, improved cognitive and motor functions, reduced severity of metabolic disorders and tumor incidence (Schroeder and Colwell 2013; Anisimov et al. 2012; Carocci et al. 2014). In LL, melatonin or its receptor agonist restored entrainment (Li et al. 2004). Importantly, melatonin may play a key role in healthy ageing due to its antioxidant and anticancer properties. Melatonin has been revealed to reduce inflammation, oxidative stress, neuron loss and mitochondrial impairment in brain and improve cognitive performance in SAMP8 mice (Morley et al. 2012). In addition, several studies proved melatonin anti-aging effect (Anisimov et al. 2012; Claustrat et al. 2005a, b). It is not clear whether melatonin prolongs lifespan because of its chronobiological or antioxidant effects, or it has complex mechanisms of action.

To sum up, CRs environmental disturbance or disorders in circadian system that occur with age or accompanies age-related diseases may be corrected by scheduled exercise, feeding, mild stress exposure or by chronobiotics treatment.

5.7 Conclusions

Mammalian circadian system comprises multiple cellular oscillators entrained and synchronized with each other and external time by the central pacemaker in the SCN which receives photic nonvisual stimuli from the retina. Synchronization of peripheral rhythms by SCN is implemented by neural signals and hormones level, particularly melatonin, regulation.

With aging, endogenous circadian disruption occurs. Circadian system exhibits diminished sensitivity to external cues and ability to adapt (entrain) external rhythm shifts, undergoes amplitude reduction and rhythms fragmentation. Anatomically, reduction in retinal ganglion cells and pineal gland innervation are observed. SCN neurons lose synchrony in firing and clock genes expression. In some tissues clock genes expression is disrupted with age.

Circadian disruption is related to aging as (a) some strains of senescence-accelerated mice have CR disruption; (b) CGs mutant mice exhibit early age-related changes; (c) circadian disruption increases incidence and severity of age-related diseases.

Some interventions may help to rescue CR in old animals, though their circadian system is less sensitive and entraining stimuli should be stronger than normal physiological cues. Some pharmacological agents influence both CR and aging.

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Chapter 6
The Circadian System and Aging of *Drosophila*

Jadwiga M. Giebultowicz

Abstract Circadian clocks generate daily rhythms in gene expression, cellular functions, physiological processes and behavior. The core clock mechanism is cell-autonomous and consists of molecular negative feedback loops that turn over with an endogenous circa 24 h period. While daily oscillations in the activity of clock genes and proteins are well understood in young fruit flies *Drosophila melanogaster*, much less is known about how the clock mechanism changes during organismal aging. Emerging data suggest that aging is associated with reduced expression of some core clock genes in peripheral head clocks, while a similar reduction may not occur in central clock neurons regulating behavioral rhythms. Clock-controlled processes also change with age. Similar as in humans, rest/activity rhythms tend to weaken with age in fruit flies, suggesting conservation of aging-related circadian impairments. The importance of circadian clocks for healthy aging is supported by observations that their genetic or environmental disruption is associated with reduced healthspan and lifespan. For example, arrhythmia caused by mutations in core clock genes lead to symptoms of accelerated aging in both flies and mammals, including neurodegenerative phenotypes. Despite the wealth of descriptive data, the mechanisms by which functional clocks confer healthspan and lifespan benefits are poorly understood. Recent studies in *Drosophila* discussed here are beginning to unravel causative relationships between circadian system and aging. They also suggest that clocks may be involved in inducing rhythmic expression of specific genes late in life in response to age-related increase in oxidative stress. The goal of this chapter is to summarize modest insights that were so far made into links between circadian system and aging and to illuminate the power of *Drosophila* for future mechanistic research in this important area.

Keywords Clock genes · Clock-controlled genes · Genome-wide gene expression · Lifespan · Healthspan · Neurodegeneration

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6.1 The Circadian System in *Drosophila* Throughout Lifespan

6.1.1 Clock Mechanism in Young Flies

Circadian clocks are cell-autonomous molecular feedback loops that generate daily rhythms in gene expression, cellular functions, physiological processes and behavior. Early observations determined that daily rhythms are not simply a response to day/night cycles but can persist in constant darkness with a "circa" 24 h period, suggesting their endogenous nature (Pittendrigh 1960). The genetic basis of circadian rhythms was convincingly demonstrated by the discovery of the first clock gene in *Drosophila melanogaster* aptly named *period* (*per*) (Konopka and Benzer 1971). A null allele of this gene resulted in loss of rhythmic behaviors, while missense alleles resulted in flies with short (~19 h) or long (~29 h) free-running periods (Konopka and Benzer 1971). Subsequent studies in *Drosophila* and mice led to the discovery of several other conserved core clock genes that form transcriptional-translational negative feedback loops that turn over every ~24 h (Hardin and Panda 2013). The simplified version of the core feedback loop in *Drosophila* is shown in Fig. 6.1. Two transcription factors encoded by the genes *Clock* (*Clk*) and *cycle* (*cyc*) form the positive limb of the clock by binding to the E-box sequences in the promoters of *period* (*per*) and *timeless* (*tim*) genes and stimulating their transcription in the early night. PER and TIM proteins accumulate in the cell nuclei late at night and repress CLK-CYC activity, resulting in the suppression of *per* and *tim* transcription until the repressive PER and TIM are degraded and the positive clock limb can restart (Hardin 2011). Clock oscillations are additionally enhanced via posttranslational modifications of clock proteins, especially via sequential phosphorylation (Hardin 2011). While clock oscillations persist in experimental conditions of constant darkness (DD), they are normally entrained to daily light/dark (LD) cycles via light-sensitive CRY protein encoded by the *cryptochrome* (*cry*) gene. Upon activation by light, CRY binds to TIM leading to its degradation. Because TIM stabilizes PER, the latter is also degraded within few hours of lights-on (Fig. 6.1). Most core clock genes are highly conserved between insects, such as *Drosophila*, and mammals (Hardin and Panda 2013). However, while flies have a single copy of each core clock gene, mammals often have two or more paralogs that are partially redundant, so that both have to be knocked down to make an animal arrhythmic (Lowrey and Takahashi 2011).

6.1.2 Organization of the Circadian Systems

Existence of the clock in specialized neurons has been established long ago based on observations of behavioral rhythms in sleep/activity, feeding and cognitive functions. More recently, chronobiologists came to the realization that instead of a

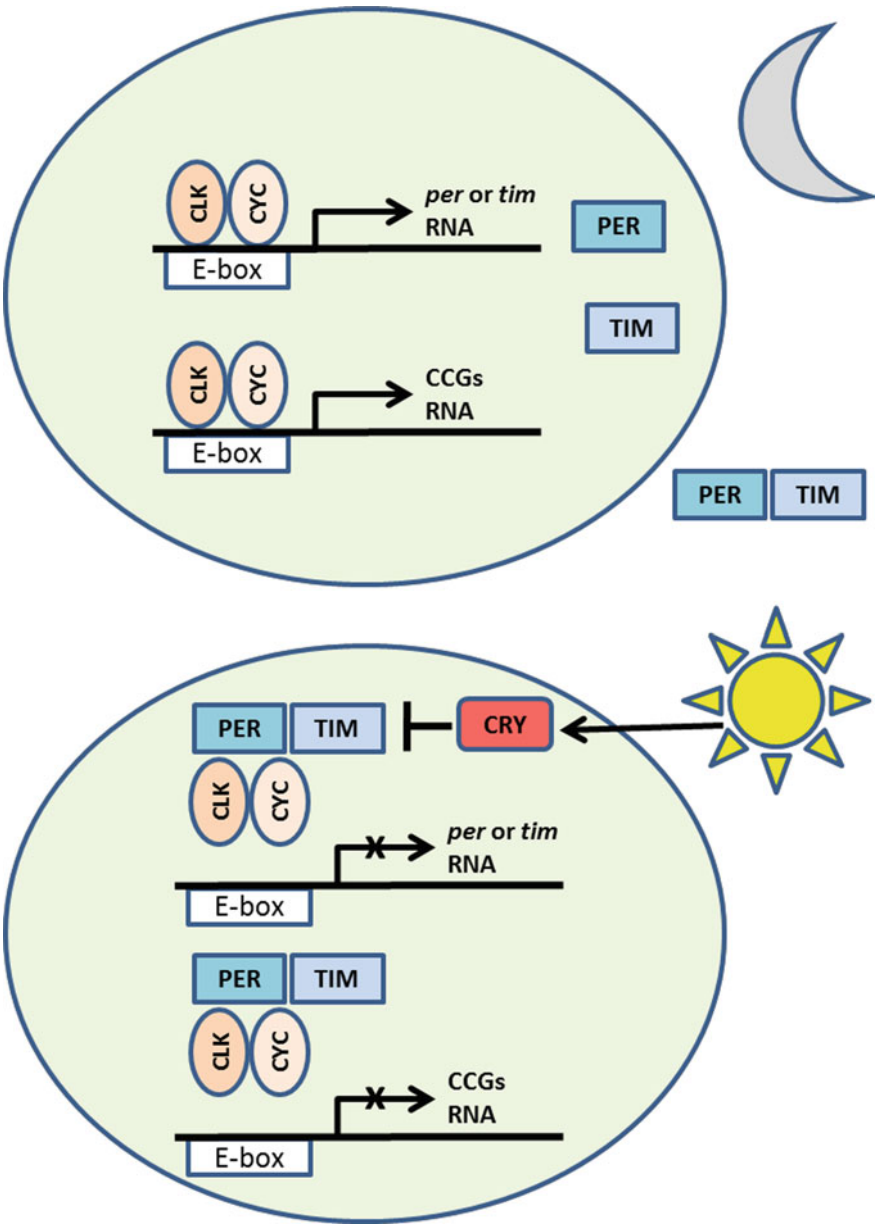

Fig. 6.1 Scheme depicting negative feedback loop that forms the core mechanism of the *Drosophila* clock. At night (*upper panel*) CLK/CYC heterodimer bind to E-box sequences in *per* and *tim* promoter and activate transcription of these genes. Resulting PER and TIM proteins form heterodimer, enter the nucleus and bind to CLK/CYC preventing further transcription of *per* and *tim*. Morning light activates CRY protein (*lower panel*) which binds to TIM causing its degradation. PER, which was stabilized by TIM, also degrades ending repressive phase of the clock and allowing positive arm of the clock to restart. Many clock-controlled genes (CCGs) also contain E-box in their promoters and are directly stimulated by CLK/CYC. Some of these CCGs encode transcription factors, which generate rhythmic transcription of additional CCGs

single "clock," animals possess multi-oscillatory circadian systems with master clocks residing in the central nervous system and peripheral clocks in cells forming most tissues. The existence of peripheral clocks was first demonstrated in insects (Giebultowicz et al. 1989; Giebultowicz and Hege 1997) and later in mammals (Balsalobre et al. 1998). The circadian master clocks in the brains of mammals and insects show anatomical and functional similarities. Both master clocks are composed of multiple neurons, which are organized in populations with different morphology sub-serving different functions (Helfrich-Forster 2004). While the mammalian central clock resides in the suprachiasmatic nucleus (SCN), the fly central clock consists of the network of pacemaker neurons controlling different aspects of sleep-activity rhythms (Nitabach and Taghert 2008). In addition to this central clock, *bona fide* clock mechanisms reside in a multitude of peripheral cells such as retinal photoreceptors, olfactory and gustatory neurons, glial cells, and fat bodies, as well as gut and excretory epithelia (Giebultowicz 2001; Cheng and Hardin 1998; Tanoue et al. 2004; Xu et al. 2008; Chatterjee and Hardin 2010; Ng et al. 2011). These clocks are called peripheral because they do not contribute directly to the behavioral sleep/activity rhythms, which persist in flies displaying clock function exclusively in central pacemaker neurons (Frisch et al. 1994; Chow et al. 2016). In contrast to mammals, peripheral clocks in flies are directly entrained by LD cycles via the CRY photoreceptive protein interacting with and degrading TIM protein. There is ample evidence that peripheral clocks can function independently of the central clock in flies (Giebultowicz 2004); however, a recent study showed that, similar to mammals, the fly central clock can regulate rhythms in peripheral tissues. It was reported that rhythmic expression of some of the fat cell genes does not depend on the local clocks, but rather on the subset of central pacemaker neurons signaling systemically via neuropeptide F released from these cells (Erion et al. 2016).

6.1.3 *Aging Alters Activity Rhythms and Circadian Clockwork in D. melanogaster*

Aging is associated with a loss of temporal coordination in vital functions. A well-known symptom of old age in mammals is the weakening of rhythmic behaviors, such as circadian sleep/wake rhythms (Kondratova and Kondratov 2012). After it was established that rest in flies is a sleep-like state (Hendricks et al. 2000; Shaw 2003), weakening of sleep/wake rhythms was reported also in aging *Drosophila* (Koh et al. 2006). These findings highlighted *Drosophila* as a model for studying age-associated sleep fragmentation and breakdown of locomotor rhythm robustness. Other features that make flies a great model to study the reciprocal relationship between aging, sleep, and the circadian system include their short lifespan (in the range of 50–80 days), excellent tools to manipulate gene expression, and conservation of molecular pathways regulating both circadian system and aging between flies and mammals.

Flies, like humans, are active during the day and sleep at night. While young flies have long consolidated bouts of sleep at night, their aging is associated with more frequent and shorter sleep bouts and declining strength of overall rest/activity rhythms (Koh et al. 2006). Subsequent studies provided further evidence for age-related decay of rest/activity rhythms, such as lengthening of the free running circadian period and an increasing percentage of flies becoming weakly rhythmic or arrhythmic with age (Luo et al. 2012; Rakshit et al. 2012; Rezaval et al. 2008; Umezaki et al. 2012). Interestingly, very old flies show drifts in the length of the free running period with females exhibiting increased incidences of phase instability and arrhythmicity in constant darkness compared to males of the same age (Luo et al. 2012).

The dampening of rest/activity rhythms could be caused by age-related changes in the expression of core clock genes, or changes in clock-mediated downstream pathways regulating sleep/activity rhythms. To address the first possibility, two studies compared diurnal rhythms of clock gene expression in heads of young and old flies collected around the clock. The clock genes *per* and *tim* showed reduced oscillations in heads of old flies compared to young, and similar changes were detected in clock-associated genes *Pdp1ε, vri,* and *cry* (Luo et al. 2012; Rakshit et al. 2012). Similar to mRNA, levels of PER and TIM proteins were shown to decline with age in both studies (Luo et al. 2012; Rakshit et al. 2012). Decreased clock oscillations in heads suggest that peripheral clocks are affected, as they form a bulk of clock-containing cells in fly heads. Indeed, it was determined by immunocytochemistry that retinal photoreceptor cells in the compound eyes of old flies have reduced expression of nuclear PER at the expected peak compared to young flies (Luo et al. 2012; Rakshit et al. 2012). However, behavioral activity rhythms are governed by the central rather than peripheral clocks; therefore, PER and TIM profiles were measured specifically in the central clock neurons. One study reported that PER maintained strong rhythms in these neurons (Luo et al. 2012), while another study showed reduced PER and TIM oscillations in the central pacemaker neurons in old flies (Umezaki et al. 2012). These differences may reflect the fact that aging rate and lifespan can show substantial variations dependent on the genetic background of the strain tested and environmental factors. Another age-related change observed in the central clock network was slight (Luo et al. 2012) or more severe (Umezaki et al. 2012) reduction in the levels of Pigment Dispersing Factor (PDF), which is released from the terminals of the PDF-positive pacemaker neurons and stimulates PDF receptor in other pacemaker neurons to form a neuronal network regulating different aspects of sleep/activity rhythms (Helfrich-Forster 2005).

Finally, it should be mentioned that aging accelerates dampening of clock gene oscillations in constant conditions both in flies (Luo et al. 2012) and mice (Nakamura et al. 2015). The latter study found that the amplitude of PER2::LUC rhythms differed only slightly between SCN explants from young and aged animals under LD conditions, while under DD conditions, the PER2::LUC rhythms of aged animals showed markedly lower amplitudes as the rhythms of individual cells rapidly became desynchronized. Altogether, these data suggest that aging degrades

circadian network properties resulting in decline in pacemaker robustness that could contribute to age-related sleep and circadian disturbances.

In the fly studies discussed above, clock gene expression was measured by quantitative RT-PCR relative to the expression levels of a reference gene. We recently used more accurate and direct technology by way of RNA sequencing (RNA-seq) to compare clock gene expression in heads of young and old female flies around the clock (Kuintzle et al. 2017). In agreement with previous qRT-PCR studies in heads of male and female flies (Luo et al. 2012; Rakshit et al. 2012), clock gene *tim* showed a decline in peak expression but remained highly rhythmic throughout aging. However; in contrast to prior qPCR data (Luo et al. 2012; Rakshit et al. 2012), RNA-seq revealed that peak expression of *per* mRNA increase with age, and this was also confirmed by qPCR. On the other hand, PER protein levels measured in whole heads by Western blot decreased significantly in old flies (Kuintzle et al. 2017), consistent with previous reports (Luo et al. 2012; Rakshit et al. 2012). Since PER repression of CLK-CYC activity reduces *per* transcription (Fig. 6.1), diminished repression due to PER deficiency could result in the observed increase of *per* mRNA. However, one would expect *tim* mRNA to increase as well but this was not observed. In summary, the opposite age-dependent changes in *per* and *tim* mRNA levels suggest that the core circadian mechanism known in young flies is altered during aging; nevertheless, the clocks remain operational throughout fly lifespan.

6.2 Functional Clocks Support Healthy Aging in Flies

6.2.1 Effects of Clock Disruption on Lifespan and Healthspan

Chronic disruptions of clock function, which can be achieved by mutations in core clock genes or perpetual environmental jetlag, tend to accelerate physiological and cognitive aging in mammals (Reddy and O'Neill 2010; Kondratova and Kondratov 2012). In addition, a null mutation in the core clock gene *Bmal1* (homolog of *cyc* in flies) significantly shortens lifespan in mice (Kondratov et al. 2006) while mutations in other clock genes had variable effects on lifespan but, in general, accelerated various age-related pathologies (Yu and Weaver 2011). Several studies demonstrated that longevity and healthspan are also negatively affected in flies with disrupted clocks [summarized in Table 6.1; for review see (Giebultowicz and Long 2015)]. In one study, flies kept under constant darkness were categorized as rhythmic or arrhythmic and it was shown that the rhythmic flies lived significantly longer than the arrhythmic ones (Kumar et al. 2005). Experiments involving flies with mutations in core clock genes lend further support to detrimental effects of circadian dysfunction on aging. For example, shortened lifespan was reported in per^s mutants with endogenous free-running period of $\sim$19 h that were maintained in mismatched external 24 h period of 12 h light/12 h dark cycles (Klarsfeld and Rouyer 1998).

Reduced lifespan was also reported in *cyc*-null males (Hendricks et al. 2003) consistent with similar effects in mice upon loss of function of the *cyc* homologue, the *Bmal1* gene (Kondratov et al. 2006). In addition to lifespan, the healthspan of flies is affected by disruption of circadian clocks. To test healthspan in clock-disrupted flies, we exposed middle-age *per*-null (per^{01}) mutants or control flies to 24-h oxidative stress (100% oxygen) and monitored their survival. While none of the flies died during 24-h of hyperoxia, subsequent mortality rates increased significantly in per^{01} mutant flies compared to age-matched wild-type flies exposed to this stress (Krishnan et al. 2009). This suggests that aging flies with disrupted clock have reduced ability to survive homeostatic challenge during aging. Additional experiments confirmed that per^{01} mutants are physiologically older when tested at the same chronological age as control flies with functional clocks. For example, the per^{01} mutants show significantly higher accumulation of oxidatively damaged proteins and lipids along with accelerated loss of vertical climbing ability (Krishnan et al. 2009). Poor climbing ability in per^{01} flies was associated with increased neurodegeneration (Krishnan et al. 2009), suggesting that clocks may have neuroprotective functions during aging. This notion was further supported by testing neurodegeneration-prone mutants *sniffer* (*sni*) (Botella et al. 2004) and *swiss cheese* (*sws*) (Kretzschmar et al. 1997) in *per*-positive and per^{01} backgrounds. Both *sni* and *sws* mutants with disrupted clocks (per^{01} background) exhibited shortened lifespan and more severe neurodegeneration at a younger age compared to either *sni* or *sws* single mutants with normal clock function (Krishnan et al. 2012). Together, these results suggest that possessing a functional circadian clock may play neuroprotective roles during aging, presumably by coordinating temporal homeostasis in the aging brain. Similarly, recent evidence in mammals suggests that circadian disruption contributes to the neurodegenerative pathologies (Musiek 2015; Ali et al. 2015).

Table 6.1 Summary of results showing negative relationships between loss of clock function and healthspan and/or lifespan in *Drosophila*

Experimental paradigm	Aging phenotypes	References
Mismatch between internal and external period in per^{s} mutants	Shortened lifespan in males	Klarsfeld and Rouyer (1998)
Loss of clock function in cyc^{01} mutants	Shortened lifespan in males	Hendricks et al. (2003)
Loss of clock function in per^{01} mutants	Higher accumulation of oxidative damage and neurodegeneration Increased susceptibility to oxidative stress Decline in climbing ability	Krishnan et al. (2009)
Loss of clock function (per^{01}) neurodegeneration-prone mutants	Shortened lifespan in males Accelerated neurodegeneration Decline in climbing ability	Krishnan et al. (2012)
Loss of clock function (tim^{01} or per^{01}) under dietary restriction	Shortened lifespan in both sexes Decreased starvation resistance Deregulated fat metabolism	Katewa et al. (2016)

6.2.2 The Circadian System Is Involved in Anti-aging Interventions

As discussed above, accumulating evidence links disruptions of the circadian system with accelerated aging; however, molecular bases of these links are poorly understood. This question has been recently addressed by investigating whether functional clocks are involved in known life-extending pathways (see Table 6.2). One of the most effective ways to promote longevity across animal taxa is by limiting nutrient availability (Bishop and Guarente 2007). Most animals alternate daily periods of activity and feeding with periods of sleep and fasting, as dictated by their circadian clocks. Under dietary restriction (DR) or time-restricted feeding, the metabolism is switched into the "fasting mode" which is associated with lifespan extension (Longo and Panda 2016). Circadian clocks regulate several metabolic and signaling pathways in flies (Xu et al. 2008, 2011; Seay and Thummel 2011; Erion et al. 2016); therefore, they could be required for nutrient related longevity effects. A recent study addressed this question and showed that functional clocks are necessary for the lifespan responses to low protein DR in *Drosophila* by demonstrating that a knockout of the core clock genes, *tim* or *per* abolished lifespan extension by DR (Katewa et al. 2016). Consistent with the involvement of the circadian mechanism, DR increased the amplitude of cycling in most circadian clock genes (Katewa et al. 2016) suggesting that the clock mechanism becomes "rejuvenated" under low nutrients. How does the clock help achieve longevity? The lifespan extension appears to be mediated through enhanced fat turnover, and lipidomic analysis suggests a role of *tim* in cycling of medium chain triglycerides under DR (Katewa et al. 2016). These findings identify a critical role for clock genes in modulating the effects of nutrient manipulation on fat metabolism and aging via regulation of clock-controlled target genes.

Another study investigated the effects of time-restricted feeding (TRF) on neural, peripheral, and cardiovascular physiology in *Drosophila*. TRF flies, which had access to food during 12 h of light, were compared to flies that had constant ad libitum (AL) access to food (Gill et al. 2015). Improved sleep patterns, prevention of body weight gain, and deceleration of cardiac aging were observed under TRF relative to age-matched AL flies, despite that total caloric intake and activity levels were similar in both groups of flies. Importantly, these effects are dependent on a functional clock, as the imposition of TRF was insufficient for protecting against cardiac aging in flies with disrupted clock function (Gill et al. 2015).

A nutrition restricted state can be mimicked by manipulating nutrient-responsive signaling pathways (Bishop and Guarente 2007), and this strategy was used to inquire whether reduced TOR or insulin signaling can reverse age-related sleep degradation in flies (Metaxakis et al. 2014). Indeed, lowering the activity of the insulin/insulin-like growth factor (IIS)/TOR signaling network improved sleep quality, with increased night sleep and day activity and reduced sleep fragmentation in aging *Drosophila* (Metaxakis et al. 2014). The question of whether or not improved sleep patterns required an intact circadian system was not addressed in

this study; however, it is known that manipulation of the insulin signaling pathway affects the free running period of the central circadian clock, and consequently, periodicity of behavioral rhythms in *Drosophila* (Zheng and Sehgal 2010). Altogether, these studies suggest that a cross-talk between healthspan-extending dietary interventions and functional circadian clocks may delay aging phenotypes in *Drosophila*.

6.3 Can Boosting the Clock Components Slow Down Aging?

Aging in flies is associated with reduced expression of specific clock genes at the mRNA or protein level (Luo et al. 2012; Rakshit et al. 2012; Umezaki et al. 2012). An important question that emerged from these studies is whether reduced expression of clock genes is causally linked to the decay of rest/activity rhythms and other aging symptoms (Table 6.2). Two studies addressed this by examining whether overexpression of specific clock genes that are reduced in aging flies could slow down deterioration of rest/activity rhythms. The first study focused on the PDF peptide, which is necessary for maintaining robust central clock network; however, its levels are reduced during aging (Umezaki et al. 2012). Overexpression of PDF specifically in the PDF-positive neurons partially rescued behavioral rhythms in old flies and shortened their free-running periods, causing apparent rejuvenation of these rhythms (Umezaki et al. 2012). In addition, PDF overexpression increased TIM (but not PER) expression in PDF-positive central clock neurons suggesting that not only output rhythms but also the clock mechanism may became stronger in these flies (Umezaki et al. 2012). The authors conclude that the age-associated reduction of PDF levels contribute to the decay of intercellular communication in the circadian network leading to the impairment of rest activity rhythms.

Another gene that appears to be significantly reduced with aging at the mRNA and protein level is *cryptochrome* (*cry*) which mediates entrainment of clock oscillations to light (Rakshit and Giebultowicz 2013). This gene encodes a blue light sensitive photoreceptive protein CRY, which leads to degradation of TIM when activated by light (Fig. 6.1) (Hardin 2011). CRY is also required in peripheral clocks for their free-running oscillations in constant darkness (Ivanchenko et al. 2001; Krishnan et al. 2001). Overexpression of CRY in all clock cells using the binary GAL4/UAS system strengthened rest/activity rhythms in constant darkness late in life when rhythms were disrupted in most control flies (Rakshit and Giebultowicz 2013). Importantly, flies with elevated CRY levels showed better climbing ability and decreased oxidative damage pointing to their delayed physiological aging (Rakshit and Giebultowicz 2013). Interestingly, overexpression of *cry* in PDF-positive central clock neurons alone was not sufficient to restore rest/activity rhythms suggesting that peripheral clocks play an active role in

Table 6.2 Summary of results demonstrating positive relationships between treatments enhancing clock function and decelerated aging in *Drosophila*

Experimental paradigm	Aging phenotypes	References
Time-restricted feeding	Slower cardiac aging Improved sleep/activity rhythm	Gill et al. (2015)
Overexpression of PDF in pacemaker neurons	Improved sleep/activity rhythm	Umezaki et al. (2012)
Overexpression of *cry* in all clock cells	Improved sleep/activity rhythm Decreased oxidative damage Increased climbing ability	Rakshit and Giebultowicz (2013)
Overexpression of *tim* in fat body clock cells	Increased lifespan on protein-rich diet	Katewa et al. (2016)
Overlapping entrainment by light and thermal cycles	Improved sleep/activity rhythm	Luo et al. (2012)

delaying behavioral and physiological aging (Rakshit and Giebultowicz 2013). Consistent with these findings, in the DR study discussed above, overexpression of *tim* in peripheral tissues, especially fat body, improved *tim* oscillatory amplitude and extended lifespan under ad libitum feeding (Katewa et al. 2016).

In addition to genetic manipulations, enhancement of entrainment signals can improve the quality of sleep/activity rhythms in aging flies. While light/dark cycles are the primary input adjusting the phase of clock oscillations, temperature cycles also entrain rhythms, even in the absence of light (Sehadova et al. 2009). Interestingly, it was reported that rest/activity rhythms could be enhanced in old flies by coupling light–dark cycles with a high–low temperature cycles (Luo et al. 2012). Taken together, the studies discussed above suggest that age-related decline in the strength of sleep/activity rhythms and other circadian impairments are not inevitably locked to aging; rather, they can be delayed by genetic or environmental manipulations (Table 6.2).

6.4 Effects of Aging on Clock-Controlled Processes

6.4.1 Expression of Clock-Controlled Genes in Young and Old Flies

Oscillating clock components impose rhythmic expression on a diverse set of target clock-controlled genes (CCGs). Their number continues to expand as sensitivity of detection techniques improve from microarray probes to RNA-sequencing

(RNA-seq) (Keegan et al. 2007; Rodriguez et al. 2013; Hughes et al. 2012). The CCGs encode transcription factors, metabolic enzymes, and regulators of neuronal processes and cellular redox (Wijnen and Young 2006; Hooven et al. 2009; Xu et al. 2011), and there appears to be an overlap between genes that are rhythmically expressed and those that are known to affect lifespan in flies. For example, genes encoding enzymes involved in glutathione (GSH) biosynthesis, the catalytic (*Gclc*) and modulatory (*Gclm*) subunits of the glutamate cysteine ligase, are known to affect lifespan (Luchak et al. 2007), and it was reported that expression of these genes is controlled by the circadian system in young flies (Beaver et al. 2012). Is transcription of these genes rhythmic throughout the lifespan? A recent study of GSH biosynthesis in old flies revealed that aging is associated with abolished daily oscillations and increased constitutive expression of *Gclc* at the mRNA and protein levels, leading to the significant increase in the catalytic activity of the GCL holoenzyme (Klichko et al. 2015). This may be due to the "overriding" of the clock by oxidative stress-induced pathways which can stimulate *Gclc* transcription in a clock-independent fashion in mammals (Forman et al. 2009).

The age-dependent shift in *Gclc* transcription from clock-controlled in young to constitutive overexpression in old illustrates the point that we cannot predict how rhythmic expression of clock-controlled genes in young flies may be altered by aging. This question needs to be addressed by comparing circadian transcription profiles in young and old organisms on a genome-wide level. We recently used this approach to measure diurnal gene expression profiles in heads of young and old female flies by RNA-seq (Kuintzle et al. 2017). Data analysis revealed that several hundred CCGs maintained similar rhythmicity patterns between young and old flies. However, many other CCGs showed dampened or abolished oscillations in old flies becoming constitutively high (like the *Gclc* discussed above) or constitutively low. Surprisingly, a large subset of CCGs showed significantly larger expression amplitudes or even de novo rhythmicity in old flies. A schematic representation of observed trends in diurnal gene expression based on our RNA-seq analysis is shown in Fig. 6.2. Our analysis revealed that stress-response genes were enriched among transcripts with age-induced rhythmicity, and subsequent experiments demonstrated that exogenous oxidative stress can induce rhythmic expression of these genes in young flies (Kuintzle et al. 2017). These data suggest that late life activation of stress responsive genes may be a strategy by which the clock helps organisms adapt to their changing cellular environments during aging. In summary, our study revealed dynamic reprogramming of the subset of circadian transcriptome including unexpected outcomes, such as age-related gain of rhythmicity in expression of several genes. Interestingly, a recent postmortem study of gene expression in the human brain reported increased rhythmicity in some daily transcript levels in the $\geq$60-yr-old group than in the <40-yr-old group, suggesting potential conservation of the age-related gain of transcriptional rhythmicity (Chen et al. 2016). Further studies are needed to determine whether late life transcriptional rhythmicity of these genes is regulated by the circadian clock and what is the functional significance of these oscillations. Nevertheless, these data suggest that the lack of knowledge how circadian transcriptome is affected by aging on a

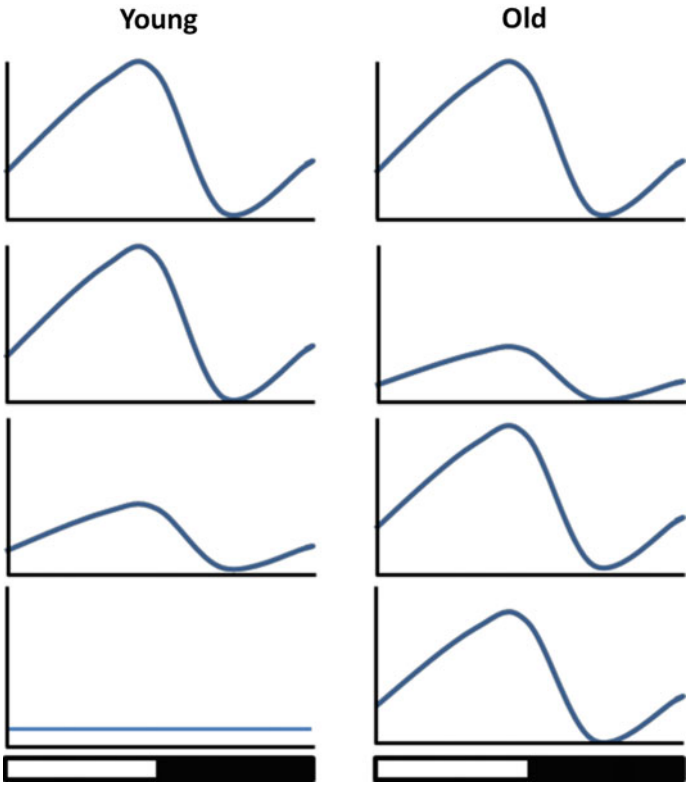

Fig. 6.2 Comparative analysis of diurnal transcriptome in heads of young and old flies revealed several age-dependent trends in gene expression. Several hundred genes that were rhythmic in young flies remained rhythmic in old, while approximately 50 genes showed significantly reduced oscillatory amplitude or loss of rhythmicity with age. Surprisingly, a subset of genes showed age-dependent gain of oscillatory amplitude or de novo rhythmicity. Scheme based on data obtained by RNA-seq in author's laboratory (Kuintzle et al. 2017)

genome-wide scale is one of the roadblocks in understanding reciprocal relationship between circadian system and aging.

6.4.2 Tissue Specific Circadian Rhythms and Longevity

Circadian clocks have similar molecular mechanism across tissues; however, clock controlled genes differ widely in a tissue dependent fashion in mammals (Zhang et al. 2014) and in flies (Kula-Eversole et al. 2009). There is also evidence for crosstalk between different tissues and organs during the aging process, such that rejuvenation of one tissue may improve lifespan in *Drosophila* (Demontis and Perrimon 2010; Wang et al. 2014). Because cells of most tissues contain the clock

mechanism, the question of interest here is whether tissue-specific clocks are differentially affected by aging in flies. The method of choice to address this question is staining of cell nuclei with antibodies against TIM or PER proteins, as both of them oscillate strongly showing high expression in clock cell nuclei during the repressive phase (late night to early morning) and almost no expression during the late day (Hardin 2011). Based on the level of PER and TIM signal, it appears that clocks in retinal photoreceptors weaken with age but other peripheral clocks (for example, in fat body or excretory epithelia) show strong PER oscillations throughout aging (Luo et al. 2012; Rakshit et al. 2012). Similarly, it appears that the network of pacemaker neurons that form central clock can maintain strong oscillations of core clock genes until very advanced age; however, sleep/activity rhythms lose their strength in these flies (Luo et al. 2012).

Another example of the disconnect between the central clock and output rhythms comes from studies of transgenic Alzheimer disease model flies carrying pathogenic Aβ amyloidogenic peptides. While lifespan is reduced and sleep/activity rhythms become disrupted at young age, these flies showed strong oscillations of the clock protein PER in the central clock neurons (Chen et al. 2014; Long et al. 2014). Together, these data suggest that normal or pathological aging may weaken behavioral rhythms downstream of the central clock. Other features of the central clock, such as reduced network communication via pacemaker generated neuropeptides in flies (Umezaki et al. 2012) or neuronal output in mammals (Nakamura et al. 2012), may uncouple core clock mechanism from downstream targets that convey rhythmicity to locomotor activity and sleep.

There is increasing evidence that the health status of single tissue types may affect insect longevity. Specifically, regenerative potential of the intestinal stem cells affects aging in *D. melanogaster* (Wang et al. 2014). Clock genes are known to modulate stem cell behavior (Brown 2014) and, interestingly, the core clock gene *period*, was reported to be critical for synchronizing intestinal stem cell divisions during regeneration of damaged gut epithelium (Karpowicz et al. 2013). Together, these data suggest that the clock may be important for long-term tissue homeostasis and the ability of stem cells to self-renew. However, long term effects of the loss of clock function in fly stem cells have not been investigated. Of note, a recent study in mice showed that genetic disruption of the molecular clockwork leads to accelerated age-dependent loss of neuronal progenitor cells and decline in adult neurogenesis (Ali et al. 2015). Together these data suggest an exciting possibility of clocks supporting healthy aging via maintaining healthy populations of stem cells in different organs.

6.5 Conclusions and Future Directions

Research on the relationships between circadian clocks and aging is in an exciting exploratory phase with many questions awaiting further studies. The status of central clock neurons in old flies requires further research, but it is clear that

rest/activity rhythms can become disrupted by normal aging or by the introduction of pathogenic Aβ peptides despite strong oscillations of clock genes in the central clock neurons (Luo et al. 2012; Chen et al. 2014; Long et al. 2014). It is possible that peripheral clocks in the fly head, while dispensable for strong rest/activity rhythms in young flies may be important in old flies via their role in maintaining temporal homeostasis in the nervous system. To address these questions, future research needs to focus on age-related changes in clock genes and clock-controlled genes and processes in specific tissues, as well as their functional significance in the regulation of healthy aging.

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Chapter 7
Circadian Control of Mitochondrial Dynamics and Its Implication in Aging

David Jacobi, Florian Atger and Chih-Hao Lee

Abstract The circadian clock promotes metabolic efficiency by pre-programmed regulation of metabolic pathways in anticipation of the upcoming feeding/fasting cycle. Recent studies have suggested that the master circadian regulators *Bmal1/Clock* play an important role in controlling mitochondrial function, including oxidative metabolism and mitochondrial dynamics. The latter includes mitochondrial fusion, fission, and selective autophagy (mitophagy) that follows fission. These processes not only allow mitochondria to undergo architectural/organizational changes in response to different nutrient conditions but also ensure quality control by removing damaged components through mitophagy. Results from mouse genetic models indicate that Bmal1-dependent regulation of fission and mitophagy genes reduces oxidative stress and maintains metabolic flexibility in the liver. In addition, *aha-1*, a *C. elegans Bmal1* homologue, is required for orderly organizations of muscle mitochondria. Interestingly, *Baml1* whole body knockout mice develop premature aging phenotypes, while AHA-1 over-expression increases lifespan in worms. Given that mitochondrial dysfunction appears to be a common feature associated with aging, obesity, and related metabolic diseases, these findings implicate an evolutionarily conserved regulatory mechanism that links mitochondrial fidelity to lifespan and/or healthspan.

Keywords Mitochondrial dynamics · Mitophagy · Metabolic diseases · Oxidative stress · Electron transport chain · Circadian rhythm

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7.1 Introduction

Mitochondria are vital for energy production through oxidative metabolism (Mitchell and Moyle 1967). Their dysfunction has been associated with metabolic diseases and aging (Chan 2006), although a causal effect could not always be established. The generation of ATP is not only driven by nutrient availability but also modulated by other cellular processes designed to cope with specific metabolic needs. A unique example is the uncoupled respiration in brown adipocytes that dissipates oxidative energy as heat (Wikstrom et al. 2014). The flexibility of mitochondria to adapt to varying nutrient conditions is also mediated by architectural changes regulated by mitochondrial dynamics that includes mitochondrial fusion, fission, and selective autophagy (mitophagy). The molecular components and cellular roles of mitochondrial dynamics have been deciphered initially from studies using Saccharomyces cerevisiae and model organisms (Westermann 2010), which reveal that mitochondria morphology is plastic upon exposure to varying carbon sources or growth conditions (Egner et al. 2002). It is now well established that mitochondrial dynamics plays an important role in regulating both energy production and quality control (Liesa and Shirihai 2013; Youle and van der Bliek 2012). The relevance of mitochondrial quality control is illustrated in a number of human diseases, notably age-related neurodegeneration. Mutations in genes involved in mitophagy have been shown to associate with autosomal recessive Parkinson's disease (Valente et al. 2004; Kitada et al. 1998).

In this chapter, we will focus on the function of mitochondrial dynamics in energy metabolism. The daily feeding-fasting cycle causes fluctuations in nutrient influx, energy demand, and oxidative stress. Metabolically active organs such as the liver provide good examples where a fine balance between bioenergetic adaptation, generation of reactive oxygen species (ROS, a side product of oxidative metabolism), and removal of damaged mitochondrial components is critical. We will first review evidence supporting a role for mitochondrial dynamics in this process. As circadian rhythm is an integral part of the feeding-fasting cycle, we will discuss mechanisms through which the circadian clock controls mitochondrial dynamics and oxidative metabolism and the relevance of such regulation in aging and age-related diseases.

7.2 Cellular Function of Mitochondrial Dynamics

The machinery of mitochondrial dynamics allows mitochondria to alter their shapes in response to varying nutrient states. In cultured cells, mitochondria form elongated and fragmented networks in low and high nutrient conditions, respectively (Liesa and Shirihai 2013). This adaptation serves several important purposes, including fine-tuning metabolic efficiency, modulating oxidative stress, and maintaining quality control (Fig. 7.1).

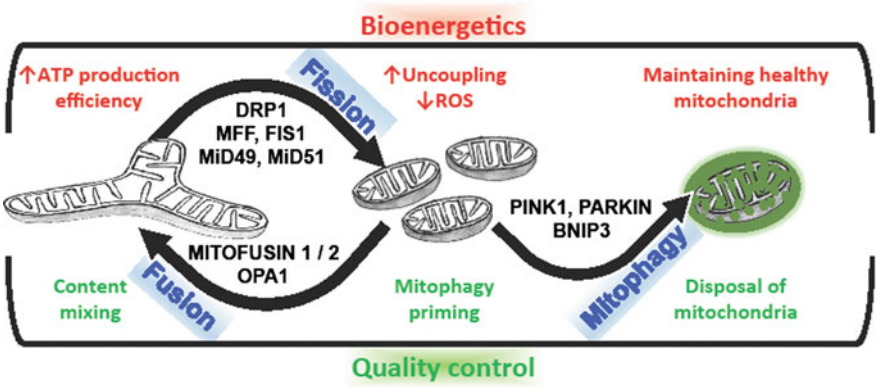

Fig. 7.1 Mitochondrial dynamics in energy metabolism and mitochondrial quality control. Mitochondrial dynamics includes the processes of fusion, fission, and mitophagy. The GTPases MITOFUSIN 1 and 2 regulate fusion of the outer mitochondrial membranes, while OPA1 fuses the inner membranes. On the other hand, Dynamin-related Protein 1 (DRP1) binds to mitochondrial receptors on the mitochondria outer membrane (e.g., mitochondrial fission factor-MFF, mitochondrial fission 1 protein-FIS1, MiD49, and MiD51) to trigger fission. Following mitochondrial fission, PINK1 and PARKIN collaborate to target mitophagy receptors to depolarized mitochondria via ubiquitylation of proteins on the outer membrane. BNIP3 could directly interact with the autophagy machinery independent of PINK/PARKIN. Mitochondrial dynamics allows mitochondria to adapt their function to meet the energy demand under different nutrient status and at the same time, modulate oxidative stress and damage

7.2.1 The Machinery

Mitochondria have a double lipid bilayer membrane. The inner membrane encloses the matrix and is folded into cristae harboring membrane-bound enzymes of the electron transport chain and ATP-synthase. Mitochondrial fission results in the separation of mitochondria in two new entities with their own matrices and intermembrane spaces. The fission machinery works through activation of a cytosolic adaptor, namely dynamin-related protein 1 (DRP1), which binds to mitochondrial receptors on the mitochondrial outer membranes (e.g., mitochondrial fission factor-MFF, mitochondrial fission 1 protein-FIS1, MiD49, and MiD51) to tether mitochondria and trigger their separation by severing both the inner and outer membranes (Chang and Blackstone 2010; Kageyama et al. 2011; Losón et al. 2013). Fusion on the other hand, brings together mitochondria in a 2-step process involving membrane embedded GTPases. Mitofusin 1 and 2 (MFN1 and 2) fuse the outer membranes so that intermembrane spaces merge (Santel and Fuller 2001; Rojo et al. 2002) and OPA1 fuses the inner membranes through which the matrices merge (Alexander et al. 2000; Delettre et al. 2000). Fusion can also be promoted by preventing fission through the inhibitory phosphorylation of DRP1 at serine 637 (Gomes et al. 2011).

Mitochondria mass is balanced by continuous degradation and biogenesis. A specialized form of autophagy targeting mitochondria, also known as mitophagy, selectively culls and degrades damaged mitochondria. The Ser/Thr kinase PINK1 and the E3 ubiquitin ligase PARKIN collaborate in this disposal. Upon mitochondria depolarization, PINK1 escapes constitutive degradation in the inner membrane by the rhomboid protease PARL and accumulates on the outer membrane. It then phosphorylates PARKIN to fully activate its ubiquitin ligase activity (Chen and Dorn 2013; Koyano et al. 2014). Activated PARKIN is recruited to mitochondria and ubiquitinates outer membrane proteins, eventually causing mitochondrial engulfment in isolation membranes to form autophagosomes that then fuse with lysosomes. The mechanisms by which PARKIN activates autophagosomes formation may involve the ubiquitin-binding adaptor P62 (Youle and Narendra 2011). In addition, BCL2/adenovirus E1B interacting protein 3 (BNIP3) is present in the outer mitochondrial membrane and can interact directly via its N-terminal region with LC3-II. This lipidated form of LC3 allows for the closure of autophagosomes that can then fuse with lysosomes to form the autolysosomes where mitochondria are being degraded.

7.2.2 *Mitochondrial Dynamics as a Sensor of Bioenergetics and Oxidative Stress*

Oxidative phosphorylation couples substrate oxidation by the electron transport chain to the generation of a trans-membrane gradient of protons that return to the matrix through the ATP synthase proton pore to generate ATP (Mitchell 1961). Because of proton and electron leaks, this coupling is incomplete. An inherent proton leak mostly mediated by the mitochondrial anion carrier proteins amounts to up to 40% of the resting metabolic rate of hepatocytes (Nobes et al. 1990). Electron leakage, on the other hand, occurs when electrons exit the electron transport chain, which react with oxygen to form ROS superoxide ($O2\cdot-$). Interestingly, proton and electron leaks are interdependent, and increasing proton leak can decrease superoxide production (Jastroch et al. 2010). As it turns out, mitochondrial dynamics seem to sense and be regulated by the redox state (Shutt et al. 2012). Mitochondrial fission is triggered by high nutrient conditions in which handling oxidative stress becomes a priority. Notably, fission increases uncoupled respiration, which may then relieve superoxide production (Liesa and Shirihai 2013). On the other hand, mitochondrial fusion and elongation occur in low nutrient conditions when cells rely more on ATP production and require higher ATP generation efficiency (Gomes et al. 2011). The physiological function of mitochondrial fusion and fission is context dependent. As mentioned above, an extreme example is seen in brown adipose tissue where energy is diverted to thermogenesis rather than ATP synthesis through fission regardless of energy status, mediated by DRP1 through adrenergic and protein kinase A (PKA) signaling (Wikstrom et al. 2014).

7.2.3 Mitochondrial Dynamics in Quality Control

About 99% of the ~1500 proteins constitutive of the mitochondrial proteome are nuclear encoded, making proteostasis key to normal mitochondria functioning. Triggering imbalance in the mito/nuclear protein ratio through inhibition of mitochondrial translation with doxycycline treatment in mammalian cells leads to decreased mitochondrial respiration and promotes mitochondrial fission (Moullan et al. 2015). In worms, inhibition of mitochondrial translation also favors mitochondrial fission (Houtkooper et al. 2013). Uncontrolled/excessive ROS production could damage mitochondrial proteins, lipids, and DNA. Damage to the electron transport chain may further exaggerate ROS production. To cope with these threats, mitochondria are gauged by several quality-control systems, including anti-oxidation enzymes, mitochondrial chaperones and proteases, and a mitochondria-associated degradation (MAD) pathway for proteins of the outer-membrane (Tanaka et al. 2010). Mitophagy differs from these processes as it selectively degrades damaged mitochondria. Several lines of evidence link mitophagy to mitochondrial quality control under stress conditions, such as altered mitochondrial proteostasis or increased oxidative stress. In Drosophila, overexpressing a mitochondrial misfolded protein leads to a mitochondrial phenotype resembling that of Pink1 or Parkin mutants, which could be rescued by Parkin overexpression (Pimenta de Castro et al. 2012). Removal of Drosophila PINK1 increases sensitivity to multiple insults including oxidative stress (Clark et al. 2006). In addition, mitochondria in *Bnip3* knockout mouse liver exhibit loss of membrane potential, abnormal structure, and reduced oxygen consumption despite increased mass (Glick et al. 2012).

The mitochondrial genome (17,000 base pairs) is more than 100,000 times smaller than the nuclear genome and encodes a limited number of proteins (17 proteins) as well as transfer and ribosomal RNAs. However, each cell contains tens to hundreds of mitochondria each containing copies of their own circular double stranded DNA genomes (mtDNA) packaged in nucleoids (Schon and Gilkerson 2010). Cells can contain one or more forms of mutant mtDNA that coexist with the wild type version, a situation termed heteroplasmy. Complementation between WT and mutant mitochondria has been demonstrated in mice with mutant mtDNA carrying an ~5000-base pair deletion (Nakada et al. 2001). Cells carrying both mutant and WT mtDNA do not show a lack of cytochrome C oxidase in any individual mitochondria, which demonstrates content mixing. This complementation would compensate for defects in mitochondria with mutant DNA. Nucleoids do not exchange DNA (Schon and Gilkerson 2010), which means mitochondria in heteroplasmic cells complement one another by sharing RNA and/or proteins. It was later demonstrated in a series of papers by Chen et al. (Chen et al. 2005, 2007, 2010) that mitochondrial fusion is the process allowing for complementation. Notably, mice with *Mfn1/2* double knock-out in skeletal muscle develop mitochondrial DNA depletion followed by severe myopathy (Chen et al. 2010).

7.3 Circadian Rhythm and Mitochondrial Activity

A major challenge faced by mitochondria is the necessity to adapt to changes in nutrient supply and energy demand resulting from feeding-fasting cycles. The circadian clock has been implicated in the control of various metabolic processes associated with the feeding/fasting responses, notably in studies using nocturnal mice that exhibit a diurnal cycle (Bass and Takahashi 2010). Not surprisingly, recent studies have demonstrated that mitochondrial dynamics and oxidative metabolism are also subjected to circadian regulation (Peek et al. 2013; Jacobi et al. 2015). This coordinated regulatory mechanism sustains metabolic flexibility through maintaining a healthy mitochondrial population and keeping oxidative stress in check (Fig. 7.2).

7.3.1 Metabolic Cycle of Oxidative Metabolism

A large fraction of mitochondrial proteins exhibits diurnal oscillations in mice, including components of the respiratory chain and enzymes of lipid and glucose metabolism (Neufeld-Cohen et al. 2016). Several mechanisms are at play. Many mitochondrial related transcripts remain constant but are translated in a rhythmic manner (Atger et al. 2015) possibly influenced by variations of poly(A)-tail length, which shows circadian fluctuations in mouse liver (Kojima et al. 2012). Mitochondrial function in mouse liver is also controlled by post-translational modification, notably acetylation (Hebert et al. 2013; Zhao et al. 2010). Mice lacking mitochondrial NAD^+-dependent deacetylase sirtuin-3 (SIRT3) have altered mitochondrial protein acetylation and impaired mitochondrial function (Hebert et al. 2013, Hirschey et al. 2011). The circadian clock regulators *Bmal1*/*Clock* modulate mitochondrial activities through transcriptional control of nicotinamide phosphoribosyltransferase (NAMPT), the rate-limiting enzyme of NAD^+ biosynthesis (Peek et al. 2013; Ramsey et al. 2009). In turn, NAD+ oscillations control SIRT3 activity to regulate mitochondrial protein acetylation (Peek et al. 2013). In concert, daily oscillations of fatty acid oxidation coincide with the rhythmic fluctuations of NAD+ levels. Accordingly, *Bmal1* KO hepatocytes show reduced fatty acid oxidation along with hyper-acetylation of SIRT3 targets. The defect of mitochondrial function is dependent on the reduction of NAD+ content in *Bmal1* KO hepatocytes, since nicotinamide mononucleotide (NMN, the product of NAMPT) supplementation restores acetylation profiles of SIRT3 targets and mitochondrial oxidative capacity (Peek et al. 2013). Rhythmic NAD + may also modulate SIRT1 activity, which then regulates metabolic transitions between fasting and feeding via the modulation of various transcription factors, including Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1-alpha (PGC1α) known to control mitochondrial biogenesis (Asher et al. 2008; Marcheva et al. 2013; Rodgers et al. 2005).

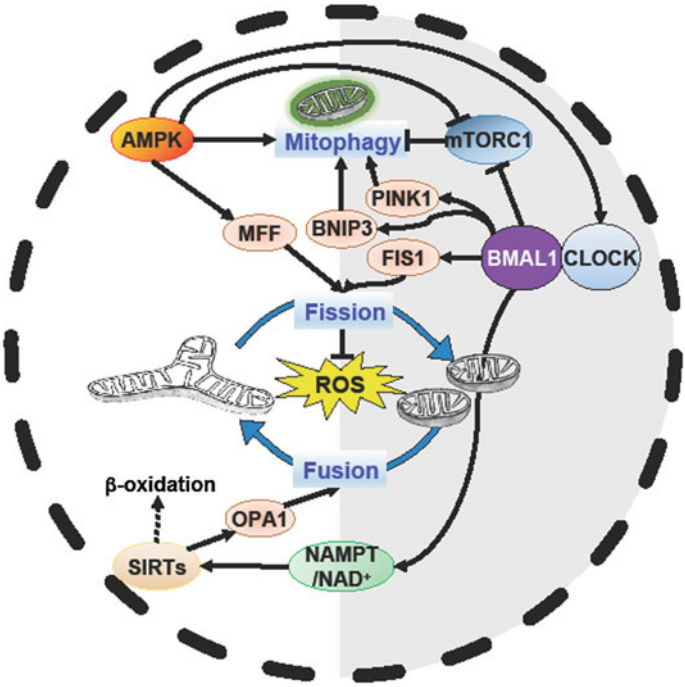

Fig. 7.2 Circadian control of mitochondrial dynamics. The circadian clock plays an important role in the control of mitochondrial function. In mouse liver, BMAL1-CLOCK regulates the expression of FIS1, PINK1, and BNIP3 in the dark/feeding cycle (shown in *grey*) to promote mitochondrial fission and mitophagy. Circadian regulation of NAD^+ level through rhythmic NAMPT expression modulates SIRT3 deacetylase activity, resulting in increased fatty acid β-oxidation in the light/fasting cycle (shown in *white*). SIRT3 has also been shown to deacetylate OPA1 to regulate mitochondrial fusion. Energy-sensing signaling pathways, including AMPK and mTORC1, control mitochondrial function, including mitophagy, by direct mechanisms or indirectly through BMAL-CLOCK. mTORC1 suppresses autophagy by inhibitory phosphorylation of ULK1, which is prevented by AMPK. AMPK also activates MFF and may therefore couple fission and mitophagy. BMAL1 has been shown to negatively regulate mTOR, while AMPK phosphorylates and destabilizes CRY1, a negative regulator of BMAL-CLOCK. Coupling of the fusion-fission-mitophagy cycle with that of fasting-feeding is critical to maintain metabolic flexibility and minimize ROS production

7.3.2 *Circadian Control of Mitochondrial Dynamics*

At the transcriptional level, circadian rhythm is controlled by a master transcription factor unit, the BMAL1/CLOCK heterodimer, whose activities are further fine-tuned by co-repressors period (PER) 1/2/3 and cryptochrome (CRY) 1/2. In the mouse liver, BMAL1/CLOCK maximal DNA binding occurs in the middle of the light cycle and the expression of target genes peaks a few hours later at the transition from light to dark cycle (Koike et al. 2012; Gong et al. 2015). ChIP-seq

analyses of commonly bound genes by BMAL1, CLOCK, and CRY1 implicate mitochondrion as a top ranked cellular component regulated by the molecular clock, suggesting that the circadian clock "anticipates" increasing nutrient influx and metabolic stress in the upcoming dark/feeding cycle by promoting mitochondrial function (Koike et al. 2012; Jacobi et al. 2015).

In addition to previously described roles of Bmal1 in oxidative metabolism and fatty acid oxidation, gene pathways predicted to be direct targets of the circadian clock also include mitochondrial fission (*Fis1* and *Mtfr1*) and mitophagy (*Pink1* and *Bnip3*). In fact, mitochondrial morphology and network in the liver determined by electron microscopy and Cox8-GFP tagging show a diurnal pattern with the light and dark cycles showing more elongated and fragmented networks, respectively. Unlike the mitochondrial network of cultured cells that changes rapidly in different nutrient conditions, this diurnal pattern persists even when food availability is transiently switched to daytime. Hepatic mitochondria of liver-specific Bmal1 knockout (LBmal1KO) mice exhibit an "enlarged" appearance throughout the day, have reduced respiration, and produce more ROS. Similarly, primary hepatocytes from LBmal1KO mice are unable to change morphology and adjust mitochondrial respiration accordingly when cultured in low or high nutrient media, suggesting that circadian regulation of Mitochondrial dynamics is key to maintain metabolic flexibility. An additional layer of circadian control of Mitochondrial dynamics could involve post-transcriptional regulation. Notably, SIRT3, whose activity is dependent on rhythmic NAD^+ levels as discussed above, deacetylates and activates OPA1 to promote fusion (Samant et al. 2014).

7.3.3 Signaling Pathways Integrating Circadian Regulation and Mitochondrial Dynamics

Adenosine monophosphate (AMP)-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) are energy-sensing molecules that regulate metabolic pathways, such as autophagy, in an opposing manner (Lamia et al. 2009; Jouffe et al. 2013). In low energy conditions, AMPK promotes autophagy through ULK1 (Egan et al. 2011). AMPK also phosphorylates and inhibits the RAPTOR subunit of mTOR complex 1 (mTORC1). mTORC1 normally prevents autophagy (Alers et al. 2012). Recent evidence demonstrates that AMPK activation triggers mitochondrial fragmentation through MFF (Toyama et al. 2016). In addition, AMPK phosphorylates and destabilizes CRY1 (Lamia et al. 2009), while BMAL1 appears to negatively regulate mTORC1 activity (Khapre et al. 2014). These findings suggest that energy-sensing pathways could directly and indirectly modulate mitochondrial dynamics through crosstalk with the molecular clock.

7.4 Mitochondrial Dynamics as a Link Between Circadian Dysregulation and Age-Related Metabolic Diseases

Aging, obesity, and related metabolic diseases appear to share a common feature-mitochondrial dysfunction. Whether this is a cause or consequence is still under debate. Nevertheless, the electron transport chain loses efficiency in aging cells with rising electron leakage and lower ATP generation (Green et al. 2011). In addition, triggering mitochondrial dysfunction accelerates aging (Kujoth et al. 2005; Vermulst et al. 2008; Trifunovic et al. 2004). Results from mouse genetic models have demonstrated that circadian dysregulation is causative to aging and age-related metabolic diseases. Bmal1 (or Clock) gene deletion at the whole body level leads to premature aging and shortened lifespan (Kondratov et al. 2006). On the other hand, depletion of Bmal1 in the liver is sufficient to cause fatty liver, dyslipidemia, and systemic insulin resistance as observed in LBmal1KO mice (Jacobi et al. 2015). These metabolic phenotypes are believed to be originated from metabolic inflexibility, as a consequence of defective mitochondrial dynamics and increased oxidative damage (Jacobi et al. 2015). It is conceivable that mitochondrial dynamics is one of the main processes that directly link circadian rhythm to aging (Fig. 7.3). In fact, AHA-1, a BMAL1-like molecule in *C. elegans*, is required for orderly organizations of muscle mitochondria and AHA-1 over-expression promotes lifespan (Jacobi et al. 2015). These results implicate an evolutionarily conserved regulatory mechanism that appears to tie lifespan or healthspan with mitochondrial fidelity.

7.4.1 Mitochondrial Integrity and Aging

As discussed above, mitochondrial fusion, fission, and mitophagy play an important role in oxidative stress handling and mitochondrial quality control. Unchecked ROS production causes oxidation of mitochondrial proteins and lipids, altering organization of electron transport chain (super) complexes and lipid composition of mitochondrial membrane. Increased mitochondrial damage and deficient turnover from impaired mitochondrial biogenesis and clearance may favor aging (López-Otín et al. 2013). mtDNA is more prone to mutations than nuclear DNA due to lack of histones, less effective repair mechanisms, and proximity to the electron transport chain where ROS is generated (Linnane et al. 1989). Although the contribution of mutations of mtDNA to aging has been controversial (Park and Larsson 2011), mtDNA mutation in human, either on protein or rRNA/tRNA coding regions, are linked to multisystem disorders that phenocopy aging (Wallace 2005). In addition, mice deficient in mitochondrial DNA polymerase G (POLG), known as mtDNA mutator mouse (Edgar et al. 2009; Hiona et al. 2010), accumulate mtDNA point mutations/deletions and have premature aging and shortened lifespan independent of ROS (Kujoth et al. 2005; Trifunovic et al. 2004; Vermulst et al. 2008).

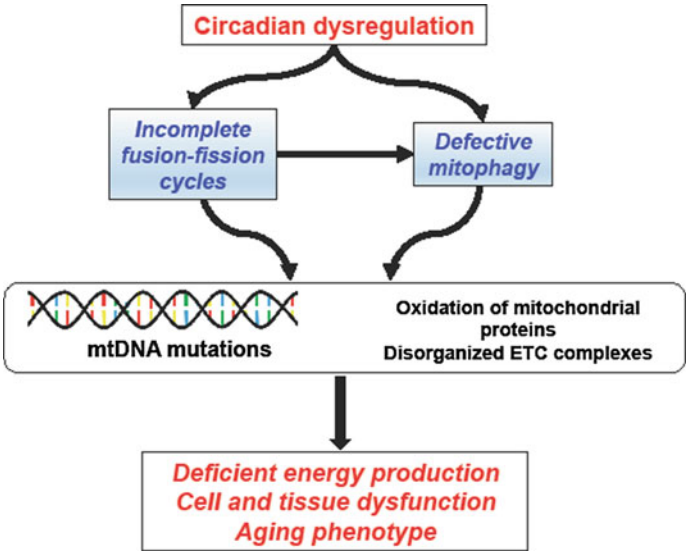

Fig. 7.3 Mitochondrial dynamics as a link between circadian dysregulation and aging. Mitochondria adapt to changes in nutrient supply and energy demand during daily feeding-fasting periods, in part, through mitochondrial dynamics. Dysregulation in circadian control of mitochondrial dynamics leads to incomplete fusion-fission cycles and defective quality control. As a result, mtDNA mutations and protein modifications accumulate, which may cause inefficient energy production and cellular dysfunction, and facilitate the aging process

7.4.2 Genetic Models of Mitochondrial Dynamics in Aging

A number of studies have provided evidence to support the notion that mitochondrial dynamics is linked to aging. In yeast, double mutants for fusion and fission display a mitochondrial network resembling that of wild-type, but have altered mitochondrial function and decreased replicative lifespan (Bernhardt et al. 2015). In contrast, promotion of fusion (or inhibition of fission) through genetic approaches results in increased stress resistance and prolonged chronological and replicative lifespan (Braun and Westermann 2011). In *C. elegans*, down-regulation of DRP1 enhanced the effect of reduced insulin signaling to extend lifespan (Yang et al. 2011). In flies, mutations in *Opa1* or *Rho-7*, both of which are required for mitochondrial fusion, greatly reduced lifespan (McQuibban et al. 2006). Interestingly, ubiquitous or neuron-specific overexpression of Parkin in adult flies increases lifespan (Rana et al. 2013), likely mediated by increased mitophagy. In the same line, genetically or pharmacologically increasing autophagy delays aging in various animal models (Ulgherait et al. 2014; Schiavi et al. 2015). In *C. elegans*, mitophagy is tied to mitochondrial biogenesis. This feedback regulation is important for stress resistance and longevity (Palikaras et al. 2015).

7.5 Conclusion

The circadian clock employs multiple regulatory mechanisms to regulate mitochondrial activity. The coordinated control of the processes of fusion, fission, and mitophagy further ensures that ROS, a side product of respiration, is properly managed and damaged components are repaired. Mitochondrial biogenesis, energetics, and quality control may represent conflicting tasks (Liesa and Shirihai 2013). The temporal regulation by the circadian clock provides a mechanism for timely, sequential activation of both processes, which optimizes metabolic flexibility, keeps oxidative stress to minimum, and preserves mitochondrial integrity. Dysregulation in BMAL1/CLOCK activity (and thus circadian rhythm) leads to a reduction in mitochondrial activity and fidelity, which likely contributes to the aging process. The detailed mechanisms through which mitochondrial dynamics is involved in aging at cellular and organismal levels remain to be fleshed out. Nevertheless, identification of pathways that restore/promote robustness of mitochondrial dynamics in future work may provide new therapeutic approaches to treat age-related metabolic diseases.

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Chapter 8
Circadian Rhythms and Proteostasis in Aging

Audrey Desvergne and Bertrand Friguet

Abstract Accumulation of oxidatively damaged proteins is a hallmark of cellular and organismal aging that has been associated to impaired redox homeostasis and decreased proteostasis. Indeed, the two main intracellular protein degradation systems, autophagy and proteasome, have been shown to generally decline with age while age-related alterations of redox homeostasis has also been reported. Both antioxidant defenses and autophagy have been shown to be regulated by the circadian system, the disruption of which results in increased oxidative stress and accelerated aging. Conversely, alterations of circadian rhythmicity have been observed with aging and during cellular senescence. It has also been recently reported that proteasome activity and oxidized protein levels exhibit circadian rhythmicity in synchronized cultured mammalian cells, hence suggesting a novel role of the circadian system in regulating proteasome function. Interestingly, circadian modulation of the proteasome activity and carbonylated protein levels is no longer observed in senescent cells. The biological connections between the circadian clock and intracellular proteostasis are addressed for autophagy and the proteasomal system in this chapter. Taken together, the present data argue for a critical link between the circadian system, redox homeostasis and both autophagy and the proteasomal system, and for the occurrence of age-related deleterious effects resulting from their combined alterations.

Keywords Aging · Circadian rhythms · Proteostasis · Protein degradation
Autophagy · Proteasome · Reactive oxygen species · Protein oxidation
Redox regulation · Signaling pathways

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8.1 Introduction

Cellular viability and functionality is maintained by the amount of proteins determined by the balance between its biosynthesis and its degradation. For humans, the daily protein requirements are lower than the synthesis which represents 300–500 g per day. Moreover, this synthesis is not affected by starvation conditions, which means that most of the proteins are synthesized from amino acids derived from degradation of pre-existing proteins. Although many studies have been devoted to the biosynthesis of proteins, the inverse process has long remained untested despite the work of R. Schoenheimer who showed in 1942, by labeling proteins with radioactive isotopes that they were constantly synthesized and degraded in animals (Schoenheimer 1942). Proteostasis has many other functions than providing of amino acids for protein synthesis. To maintain cell viability and functionality, proteins are subjected to a very precise and efficient renewal. Indeed, damaged, misfolded or modified proteins that have become "useless" and deleterious for the cell must be selectively recognized and then degraded. Furthermore, over the past three decades, it has become increasingly clear that selective proteolysis is a key mechanism not only in controlling the quality of proteins but also in their regulation. Proteostasis is therefore involved in many biological processes such as cell proliferation, cell cycle progression and more particularly in the cyclical life of the cell by intervening in the control of circadian rhythmicity. It has also a major role in aging since the accumulation of damaged proteins, which represents a hallmark of biological aging, has been proposed to be directly linked to a decrease of proteolysis activity (Carrard et al. 2002; Stadtman 2006; Chondrogianni et al. 2014). In addition, alteration of proteostasis has been found in a number of age-associated pathologies such as neurodegenerative diseases and cancers.

Several systems have made it possible to fulfill those proteostatic functions. For a long time it was assumed that most intracellular proteins are degraded in the lysosome. The lysosomal pathway involves many hydrolases including cathepsins B, D, E, H, L (Chapman et al. 1997). The discovery of the proteasome as a central mediator of the UPS (ubiquitin-proteasome system) took place in the late 1980s. This energy-dependent proteolytic system, present in the cytosol and the nucleus, is the major degradation pathway since it degrades 80–90% of intracellular proteins and it has been proven that many critical cellular events are controlled by this system. Different types of proteasome exist in the higher eukaryotes depending on their tissue localization. These proteasomes are further increasing the level of degradation specificity. Moreover, the Lon protease a specific ATP-dependent protease in the mitochondrial matrix is believed to correspond to the mitochondrial proteasome homologue for the degradation of oxidized proteins (Hamon et al. 2015). In addition to the proteasome, the Lon protease and lysosomal enzymes, calpains are calcium-dependent cytosolic cysteine proteases (Saez et al. 2006). However, the role of calpains is limited to the maintenance of cellular architecture and the activation of certain enzymes.

Proteostasis and more particularly the proteasomal system, is finely regulating certain biological processes essential for the cell, such as the circadian rhythmicity. Indeed, the degradation of key players in the biological clock is implicated in maintaining a 24-h cycle of many proteins expression and activity. Several studies have shown that the circadian clock in turn controls the activity of proteolytic systems such as autophagy and proteasome. During aging, the circadian clock dysfunction is therefore expected to affect both the regulation and activity of these intracellular proteolytic systems. Since the age-associated alteration of their activity results in deleterious consequences for the cell, the link between the circadian clock, intracellular proteolysis (autophagy and proteasome) and redox homeostasis is addressed in the context of increased oxidative stress and aging.

8.2 Autophagy and the Circadian Clock in Aging

The term 'autophagy' (or macroautophagy), coined by de Duve in 1963 (de Duve 1963), comes from the Greek words 'phagy' meaning 'eat', and 'auto' meaning 'self' and defined a system that delivers the cytoplasmic components and cellular organelles to lysosomes for degradation (Deter et al. 1967; Nakatogawa et al. 2009). Autophagy is a fundamental eukaryotic cellular process conserved from yeast to humans that controls protein and organelle degradation, and has essential roles in survival, development and cellular homeostasis. At the beginning of research on this topic a question remained outstanding: why does the cell need to self-digest its own components? The first hypothesis was that autophagy serves only as a cellular rubbish-disposal mechanism that eliminates misfolded or long-lived proteins, protein aggregates that are too large to be handled by the proteasome, superfluous or damaged organelles, and certain pathogens (Iwata et al. 2006; Ebato et al. 2008; Ueno and Komatsu 2017). Thanks to the substantial advances in the molecular studies of autophagy, that have taken place over the past 15 years, it has been shown that this process is also an adaptive response to provide nutrients and energy on exposure to various stresses. Because autophagy carries out a variety of functions it has been connected to human pathophysiology and it has also important implications in various physiological and physiopathological processes such as development, aging, immune response, cancer, diabetes, hepatic steatosis, skeletal myopathy and neurodegeneration (Ebato et al. 2008; Wong et al. 2011).

8.2.1 Molecular Mechanisms of Autophagy and Its Regulation

There are three types of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy. Autophagy, which usually refers to macroautophagy, is a general term for the sequestration inside double-membrane vesicles

and the subsequent degradation, by acid hydrolases capable of degrading proteins, nucleic acids, lipids and carbohydrates, of the cytoplasmic cargo within lysosomes (Nakatogawa et al. 2009). This process initiates with the formation of isolation membranes (or phagophore), which expand while becoming spherical, engulf cytosolic components and form enclosed vesicles composed of a double membrane-bound structure, called the autophagosome (Fig. 8.1) (Levine and Klionsky 2004). The synthesis of these double membrane-bound compartments is the most remarkable feature of autophagy that seems to be mechanistically distinct from conventional membrane traffic. The outer membrane of the autophagosome subsequently fuses with lysosomal membrane to form autolysosome, where the degradation of the contents together with the inner membrane is realized (Fig. 8.1). Different points of control are under the influence of numerous factors that regulate the autophagic flux.

The regulatory process of autophagy is divided into two distinct forms: selective that is regulated under homeostatic conditions or nonselective autophagy which is induced upon starvation or in response to external or internal stress related conditions (Klionsky and Schulman 2014). The core autophagic machinery is the same

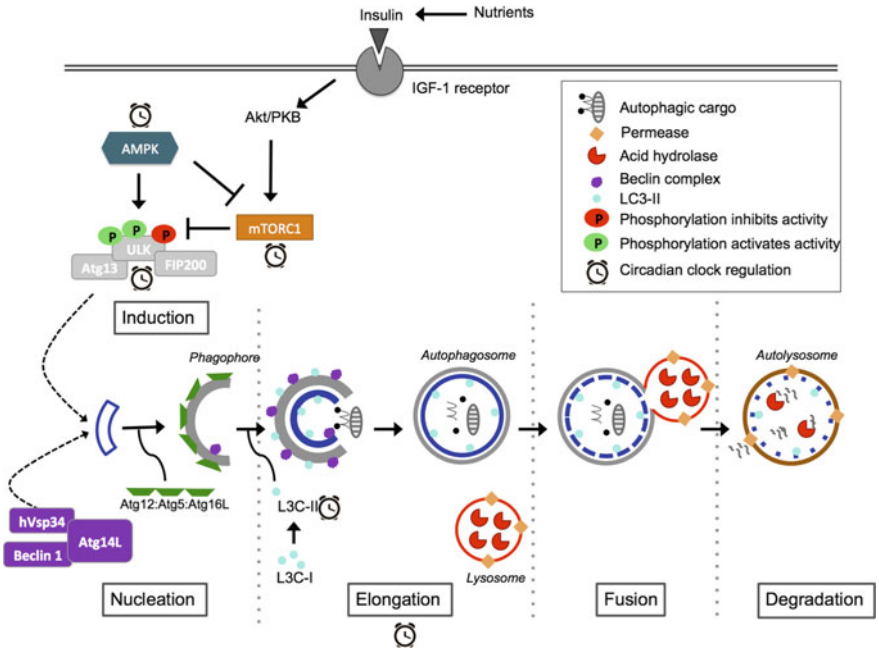

Fig. 8.1 Schematic steps of macroautophagy from nucleation of a phagophore to a mature autolysosome. Multi-protein complexes are formed at each of the five sequential steps of macroautophagy: induction, nucleation, elongation, fusion of autophagosomes with lysosomes, and degradation of the autophagic cargo. See text for details. The circadian regulation of autophagic flux represented by a clock on the figure takes place at the level of the initiation and elongation steps

in the both forms and is structured by a number of autophagy-related genes (ATG) identified in yeast almost three decades ago (Mehrpour et al. 2010). Moreover, there is remarkable conservation, from yeast to mammals, of the proteins and pathways that carry out and regulate autophagy (Yang and Klionsky 2010; Klionsky and Schulman 2014; Mehrpour et al. 2010).

There are six checkpoints of autophagy: induction, i.e. autophagosome formation into three discrete steps corresponding to nucleation, elongation and fusion, substrate targeting and degradation (Fig. 8.1). All these critical control points require a specific set of proteins (Rotter and Rothermel 2012; Chen and Klionsky 2011; Wirawan et al. 2012; Wong et al. 2011).

8.2.1.1 Induction

Formation of the autophagosome has been characterized as a small crescent-shaped structure called isolation membrane or phagophore, and is initiated by a complex consisting of the serine/threonine protein kinase unc-51-like kinase-1 (ULK1) and 2 (ULK2), the RB1-inducible coiled-coil 1 (RB1CC1; also known as FIP200, focal adhesion kinase (FAK-family-interacting protein of 200 kDa) and Atg101 (an Atg13-binding protein) (Wong et al. 2011; Yang and Klionsky 2010; Feng et al. 2014). In yeast, this complex corresponds to the Atg1 complex formed by Atg1, Atg13 and the Atg17–Atg31–Atg29 subcomplex (Feng et al. 2014; Nakatogawa et al. 2009). Autophagy induction is controlled by two master regulators of ULK, mammalian target of rapamycin (mTOR) complex1 and AMP-activated protein kinase (AMPK) (Kim et al. 2011). mTOR is a serine/threonine kinase (class I PI3 K) placed downstream of insulin signaling. It acts as a central inhibitor of autophagy by phosphorylating inhibitory sites on the unc-51-like kinase 1 (ULK1 or ULK2) (Fig. 8.1) (Jung et al. 2009). mTORC1 can be inhibited by some external stressors as nutrient starvation directly and indirectly by AMPK or by its inhibitor rapamycin. This inhibition leading to the dephosphorylation and activation of ULK1 at a specific site. The activity of ULK1 is increased thanks to its phosphorylation by AMPK. In parallel, AMPK also mediates inactivation of mTORC1, hence promoting autophagy (Tripathi et al. 2013).

8.2.1.2 Nucleation

This process consists of the nucleation and the assembly of the initial phagophore membrane (Fig. 8.1) and requires the formation of a large protein complex constituted by class III phosphatidylinositol 3-kinase (PI3 K or hVps34), its regulatory subunits p150 or hVps15, Beclin1, and Atg14L, the more recently discovered mammalian homolog of Atg14 (Klionsky and Schulman 2014; Mehrpour et al. 2010; Nakatogawa et al. 2009). The activity of this complex is controlled by several regulators but the precise mechanisms by which the ULK:Atg13:FIP200 complex interacts with the beclin1:hVps34:Atg14L complex have not been clearly identified.

8.2.1.3 Elongation

Following the nucleation step, other Atg proteins are recruited to the membrane of the phagophore to promote elongation, expansion and completion of the autophagosome (Fig. 8.1). During those steps, a conjugation between Atg12 and Atg5 is observed and then this complex interacts with Atg16L to form a multimeric complex Atg12:Atg5:Atg16L. This complex, with the participation of Atg3, Atg4 and Atg7 proteins, associates with the microtubule-associated protein 1 light chain 3 (LC3)-I, which is recruited by the beclin1: hVps34:Atg14L complex to form the mature autophagosome (Rodney et al. 2016; Klionsky and Schulman 2014; Mehrpour et al. 2010). The lipidated form of LC3 (LC3-II) promotes the translocation of LC3 from cytoplasm to the membrane of the pre-autophagosomes (Mizushima et al. 2001; Fujita et al. 2008). The presence of LC3-II on the outer and inner membranes of the autophagosome allows the induction and maturation of the autophagosome (Martinez et al. 2015; Nath et al. 2014). Then the delipidation of LC3B-II, recycling it back to LC3B-I, ensure the elongation of the autophagosome.

8.2.1.4 Fusion and Degradation

A mature autophagosome can fuse with several types of vesicles from the endosomal/lysosomal pathways including late endosomes and lysosomes (Eskelinen 2005). Indeed, it can fuse directly to a lysosome for the degradation of its contents or first fuses with an endosome prior to trafficking to the lysosome. The majority of the components involved in this process are not specific for autophagy since they are also required in vesicular trafficking in general. It is well described that autophagosome maturation and degradation require the action of late endosome marker protein Rab7 and lysosomal membrane protein LAMP-2 (Wong et al. 2011). But despite the discovery of new regulators the underlying mechanisms of this process remain unclear. Degradation of autophagic cargo is the final step controlling the autophagic flux. It takes place in the autolysome where the release by permeases of the lysosome cytoplasm allows the degradation of the substrates by acid hydrolases (Fig. 8.1).

8.2.2 Circadian Rhythms and Autophagy

8.2.2.1 Circadian Regulation of Autophagy

The description of a circadian regulation of autophagy is ancient since it dates back to the 70s. Indeed, the circadian rhythm of autophagy was initially shown by Pfeifer (1971) who demonstrated by comparative morphometric electron microscopy studies that the number of autophagic vacuoles (autophagosome formation) varies throughout the day in several tissues, including renal proximal tubules in normal

rats, cardiac muscle and meal-fed rats liver (Pfeifer and Scheller 1975, 1981). More precisely, it was shown that the autophagy process (segregation and degradation) is maximal in the light phase when rats are inactive, whereas during the dark phase it is minimal. Moreover, electron microscopy quantitative analyses carried out in the eyes of rats have shown, that in the inner segment of retina rod cells, autophagy also peaks in the light phase, about 3 h after the peak of disk-shedding (Remé and Sulser 1977). In recent studies, to determine whether autophagy is rhythmic, Ma et al. (2011) in mice and Huang et al. (2016) in zebrafish, examined molecular markers of autophagy, performed electron microscopy and expression profiling of autophagy-related genes (Ma et al. 2011; Huang et al. 2016). With all these techniques, they could have access to the different stages of autophagy after its activation i.e. the autophagosome formation, its conversion to autolysosome, and the subsequent lysosomal degradation. Immunoblot analyses of mouse liver, skeletal muscle, cardiac muscle and kidney lysates, harvested at different time points, were performed to monitor the unconjugated and the lipid-conjugated form of microtubule-associated protein 1 light chain 3 (LC3, mammalian homologue of yeast Atg8 and zebrafish Map1lc3b) present in the cytosol (LC3-I) and the autophagosome (LC3-II) respectively. The protein expression of sequestosome 1 (p62 or Sqstm1 in zebrafish), a protein that interacts with LC3 and provides autophagy cargos for lysosomal degradation was also measured (Komatsu et al. 2007). It was shown that the relative amounts of both LC3-I/II (Map1lc3b-I/II) and p62 (not Sqtm1) exhibit a robust circadian rhythmicity in mice and zebrafish larvae and liver, meaning that the conversion of autophagosome into autolysosome is rhythmic in those tissues. In order to complement these results and to have accuracy on the in vivo autophagy flux, a method using leupeptin, a lysosomal protease inhibitor, was used in mouse liver (Haspel et al. 2014). The measurement of leupeptin-induced LC3-II accumulation is the lowest in the middle of the dark phase reflecting a cyclic activation of autophagy flux in the liver. In addition using electron microscopy, they also confirmed that autophagosome formation was rhythmic and demonstrated by quantitative PCR (qPCR) analyses that the circadian clock in mouse liver regulated several genes involved in various steps of autophagy. The cyclic mRNA expression was confirmed by microarray analyses of livers from mice kept under constant darkness (Hughes et al. 2009). In yeast, during continuous growth under nutrient-limited conditions, a rhythmic expression of genes of the lysosomal pathway has also been reported (Tu et al. 2005).

Interestingly, experiments achieved with cultured murine NIH 3T3 fibroblasts synchronized by treatment with dexamethasone (Balsalobre et al. 2000) show that the circadian clock drives cell-autonomous oscillations of autophagy levels that may depend on clock gene expression levels (Kalfalah et al. 2016). Autophagy was monitored by quantification of LC3-II and rhythmic changes in LC3-II levels were observed with a period length of around 24 h. Furthermore, after lysosome inhibition LC3-II accumulation was measured and this rhythmic accumulation was in antiphase with the expression of endogenous *Bmal1* mRNA and in phase with the *Per2* mRNA expression. Moreover, after siRNA treatment of PER2 and BMAL1 a reduction of LC3-II levels was only observed after knockdown of PER2. PER2

knockdown also reduced the cellular amount of the ULK1 protein without affecting the *Ulk1* mRNA expression as well as several other key autophagy genes such as *Map1/Lc3b*. All these findings imply that the regulation of these autophagy proteins is carried out by an indirect control such as post-translational modifications that implicate PER2 but not by direct transcriptional regulation.

8.2.2.2 Transcriptional Regulation of Autophagy Rhythmicity

The molecular mechanisms underlying circadian regulation of autophagy are not clearly understood yet. Indeed, while food intake resets the phase of autophagy rhythmicity, it remains unknown whether it is the same mechanism as for the peripheral clock synchronization. Rhythmic expression of autophagy genes suggests that the transcriptional regulation of autophagy will be the most likely way. Functional analyses in mice of transcription factors and cofactors known to regulate mammalian clock and/or hepatic starvation response, including Bmal1, RORα, RORγ, Crtc2, C/EBPα, C/EBPβ, BAF60a, BAF60c, and PPARα, identified C/EBPβ as a key regulator that links autophagy to circadian pacemaker and maintains nutrient homeostasis throughout light/dark cycles (Ma and Lin 2012; Huang et al. 2016). C/EBPβ, a basic leucine zipper transcription factor that regulates diverse biological processes, including immune response, cell differentiation, and metabolism is under the control of both circadian and nutritional signals (Croniger et al. 2001; Zhaodan Cao and McKnight Steven 1991; Wang 2000). This transcriptional factor can alone activate autophagic protein degradation upon binding and activation of the promoters of some autophagy and lysosomal genes such as *Ulk1*, *L3C* and *p62* (Ma et al. 2011). Furthermore, a non-functional liver clock obtained by liver-specific *Bmal1* knockout mice results in the significant diminution of the amplitude of C/EBPβ mRNA oscillation and in the loss of autophagy rhythmic genes. In addition, the autophagy flux was significantly reduced. These results indicate that the molecular components of the circadian clock do not directly regulate the autophagy genes but most likely via C/EBPβ (Fig. 8.2).

Similar to C/EBPβ, the transcription factors FOXO3, sterol regulatory element binding protein 2 (SREBP2) and TFEB have been described as possible transcriptional regulators of autophagy genes (Fig. 8.2) (Mammucari et al. 2007; Xiong et al. 2012; Settembre et al. 2011; Seo et al. 2011). Indeed, FOXO3 as well as FOXO1 induces the expression of several autophagy genes in skeletal myocytes. However, it is not clear yet how these factors work together with mTOR or AMPK signaling pathways to regulate the autophagic activity. Moreover, as for C/EBPβ, how these factors contribute to circadian regulation of autophagy remains unknown. The only link found between the circadian clock and FOXO is related to insulin which can regulate the liver clock gene expression via this transcription factor (Chaves et al. 2014; Ferrell and Chiang 2015).

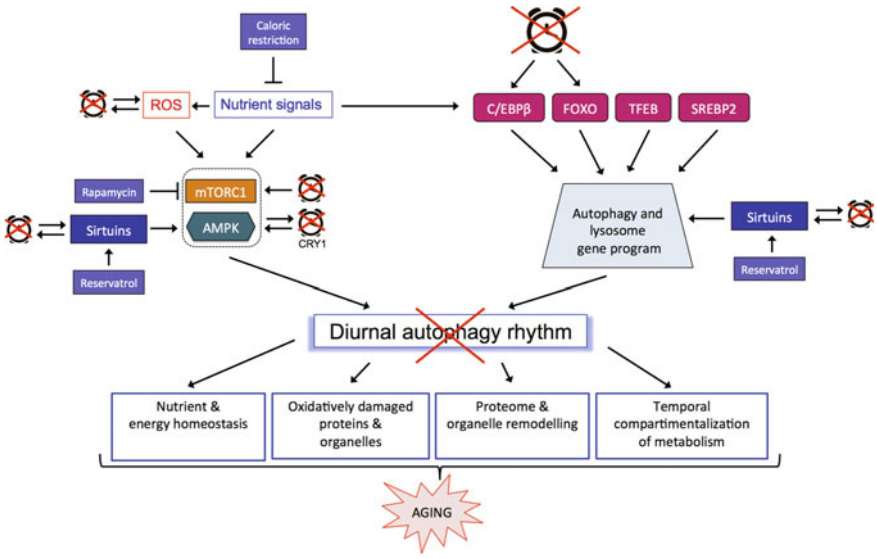

Fig. 8.2 Model of the circadian regulation of autophagy and the consequences in its impairment. Nutrient signals and ROS activate the mTOR/AMPK pathway which phosphorylates components of the ULK1-FIP200-ATG13 complex. *Purple labels* represent factors involved in both aging and autophagy regulation pathways. The circadian clock can regulate the mTOR/AMPK pathway at different levels as represented in the figure by a clock. The *double arrow* means that the regulation is mutual between the clock and particularly the Cry1 gene and AMPK. The autophagy and lysosome gene program is controlled by several transcription factors, including C/EBPβ, FOXO, SREBP2, and TFEB. Among these transcription factors only C/EBPβ and FOXO displayed a circadian rhythmicity. The impairment of the clock by the circadian clock genes knockout abolished the diurnal autophagy rhythm and has a major impact in all the functions controlled by autophagic activity. The perturbation of all these functions have been implicated in aging and are found in the normal aging process

8.2.2.3 Non-transcriptional Regulation of Autophagy Rhythmicity

The existence of a retroaction positive negative feedback loop between AMPK and the clock has been demonstrated in mouse liver. Indeed, AMPK activity and nuclear localization were exhibiting circadian rhythmicity while stimulation of AMPK by nutrient signals phosphorylates and destabilizes the clock component cryptochrome 1 (CRY1) (Lamia et al. 2009). In another study a circadian regulation of mTOR pathway was suggested with a possible link between mTOR signaling, diurnal gene expression and metabolic regulation (Loboda et al. 2009). However the role of mTOR and AMPK pathways in driving rhythmic autophagy activation in tissues remains to be further examined. Interestingly, it was shown in skeletal muscle that autophagy was regulated by reactive oxygen species (ROS) which act as cellular signal transducers in different signaling cascades depending on their localization (Rodney et al. 2016). The activation of autophagy with high levels of ROS serves to reduce oxidative stress and prevents the accumulation of oxidative damages. Among the

pathways that regulate autophagy by ROS, the mTOR and AMPK pathways have been evidenced. Different studies demonstrated that ROS activate autophagy by regulating the activation or inhibition of PI3 K/Akt/mTORC1-signaling pathway (Zhang et al. 2009; Byun et al. 2009; Talbert et al. 2013; Pal et al. 2014). AMPK activity has also been shown to be under the control of the redox balance. Indeed, the exposure of cells to H_2O_2 activates AMPK and consequently increases autophagy activity (Rodney et al. 2016). Furthermore, the existence of regulation loops between the ROS and the circadian clock has been demonstrated (O'Neill and Reddy 2011). Indeed, there is an endogenous rhythmicity of ROS homeostasis that could be implicated for controlling the circadian modulation of autophagy.

Evidence for a circadian control of such processes as nutrient metabolism and redox homeostasis makes it possible to consider that the age-associated decline of the circadian clock plays an important role in aging. Indeed, autophagy regulation by nutrient metabolism, ROS and the circadian clock suggests that the failure of autophagy and the dysregulation of the clock are closely connected in the aging process.

8.2.3 Connection Between Autophagy, the Circadian Clock and Aging

As previously shown, a link between the circadian clock and the aging process has been clearly established (Kondratov 2007; Krishnan et al. 2009; Panda et al. 2002). It is therefore tempting to speculate that both autophagy and the circadian clock represent key processes to counteract tissue degeneration and aging in many organisms. Conversely, the aging process compromises the circadian clock and autophagy. Several hallmarks of aging are related to autophagy. A common feature of aging cells is the gradual accumulation of deleterious modifications resulting in a decline in cellular function and eventually leading to cell death and disease. Damaged proteins and organelles accumulation, as carbonylated proteins and defective mitochondria, is one of the most important aging cellular events mainly caused by the decline of different catabolic functions such as proteasome and autophagy (Donati et al. 2001; Del Roso 2003; Baraibar and Friguet 2012). Moreover, failure in the clearance of proteins due to the decline of autophagy has been associated with age-related pathogenesis in neurodegenerative diseases.

The Levine group led the first experimental study implicating autophagy genes in aging, that has shown that bec-1, the *C. elegans* orthologue of yeast and mammalian autophagy gene *APC6/VPS30/ beclin1*, is essential for lifespan-extension (Meléndez et al. 2003). Then, experiments confirmed that the loss-of-function in autophagy genes as *Atg7* results in the accumulation of damaged proteins and organelles in mice (Kuma et al. 2004) and accelerates aging and shortens life span in *Drosophila melanogaster* (Juhasz et al. 2007). In contrast, the upregulation of autophagic flux by mitochondrial stress-adaptation and caloric

restriction, an intervention known to slow down aging, enhanced longevity in adult flies and rescued aged cells from accumulating dysfunctional mitochondria (Simonsen et al. 2014; Bergamini et al. 2004). Moreover, genetic and pharmacological experiments that stimulate autophagy prolong the life span of various model organisms as reported by Madeo et al. (Madeo et al. 2010).

Among other examples connecting the pro-longevity effects of autophagy and the circadian clock, Sirtuin 1, a phylogenetically conserved NAD + dependent histone deacetylase, is believed to have a predominant role in aging, and the Sirtuin 1 pathway has also been identified to induce autophagy. Indeed, transgenic overexpression experiments of *sirtuin 1* increases the longevity of *Drosophila melanogaster* (Rogina and Helfand 2004) and *Caenorhabditis elegans* (Tissenbaum and Guarente 2001) and inhibits apoptosis in mammalian cells (Cohen et al. 2004). Conversely, transgenic expression of *sirtuin 1* induces autophagy in mammalian cells in vitro (Lee et al. 2008) and in *C. elegans* in vivo (Morselli et al. 2014). Autophagy induction is thought to be mediated through deacetylation of major regulators of autophagy such as AMPK and autophagy relevant gene products such as Atg5, Atg7 and Atg8/LC3. Moreover, the knockdown, knockout or pharmacological inhibition of *sirtuin 1* prevents the induction of autophagy. In addition to be involved in aging and autophagy, sirtuins also act in the modulation of the clock. Indeed, a positive-negative feedback loop involving a circadian regulation of sirtuin activity, transcription of nicotinamide phosphoribosyl-tranferase (NAMPT) and NAD+ oscillations has been described (Imai and Guarente 2014; Sahar and Sassone-Corsi 2009). In turn, SIRT1 can inhibit the transcription of NAMPT. Since NAD+ plays a role in the integration of nutritional sensors to the circadian rhythmicity, the deregulation of the clock may affect SIRT1 and consequently autophagy activity. Another pharmacological inducer of autophagy independent of Sirtuin 1 is rapamycin that prolongs life in different organisms including mice by inhibiting TORC1 (Madeo et al. 2010). As described above, the mTOR and AMPK pathways that control autophagy activation are under ROS and circadian regulation. Hence, the deregulation of the circadian clock may explain, at least in part, the decreased autophagy activity observed during aging.

Recent studies on skin fibroblasts have shown a direct link between age related changes of clock gene expression and the decline of autophagy levels. Indeed, the expression levels of transcriptional repressor components of the circadian oscillator *PER2* are strongly reduced in primary dermal fibroblasts from aged humans, as well as autophagic flux. An altered core clock gene expression may participate in the age-related reduction of autophagic flux and promote the aging process of human skin fibroblasts. Interestingly, the dysfunctional autophagy mechanism of aged cells could be partially restored by elevating the expression of a single core clock gene *PER2*. Hence, the age-related changes of clock gene expression may promote declining autophagy levels.

8.3 The Proteasome and the Circadian Clock in Aging

To maintain cell viability and functionality, proteins are subjected to a very precise and efficient renewal. Indeed, damaged, misfolded or modified proteins that have become "useless" must be recognized and then degraded. Several systems have made it possible to fulfill this function. In eukaryotes, the proteasome is the most important one in the cytosol and the nucleus as it degrades more than 80% of intracellular proteins. The proteasome is therefore involved in many biological processes such as cell proliferation, cell cycle progression and more particularly in the cyclic life of the cell by intervening in the control of circadian rhythmicity. It has also a major role in aging since its activity is generally decreasing with age, resulting in the intracellular accumulation of deleterious damaged proteins. In addition, an altered function of the proteasome has been found in a number of age-associated pathologies such as neurodegenerative diseases and cancers.

8.3.1 The Proteasome and Its Different Regulatory Mechanisms

The 20S proteasome corresponds to the central catalytic particle and it can be associated with different regulators depending on the pathway and thus on the protein target to be degraded. Among these regulators are 19S or PA700, the 11S or PA28 and the 1Blm10 or PA200 (Fig. 8.3).

The proteasomal pathways are usually distinguished into two categories: the ATP-dependent and the ATP-independent pathways. It was long thought that the ATP-dependent pathway was the most important one but it was recently demonstrated that the ATP-independent pathway is also essential and even preferential under certain conditions (Erales and Coffino 2014; Pickering and Davies 2012a; Hwang et al. 2011). Indeed, in such a situation as oxidative stress, it is primordial to have a very fast response to eliminate damaged proteins and this is made possible by the ATP-independent pathway.

8.3.1.1 The 20S Particle: The Catalytic Core

The proteasome is an ancient enzyme that has evolved towards greater complexity of its subunits and its regulators while preserving its cylindrical architecture consisting of four stacked rings, each carrying 7 subunits of types α or β that assemble in the form of a barrel (α1-7, β1-7, β1-7, α1-7). In Archaea such as *Thermoplasma acidophilum*, the proteasome consists of two outer rings made up of seven identical α subunits and two inner rings made up of seven identical β subunits (Lowe et al. Löwe et al. 1995). Increased complexity of proteasome results, in yeast

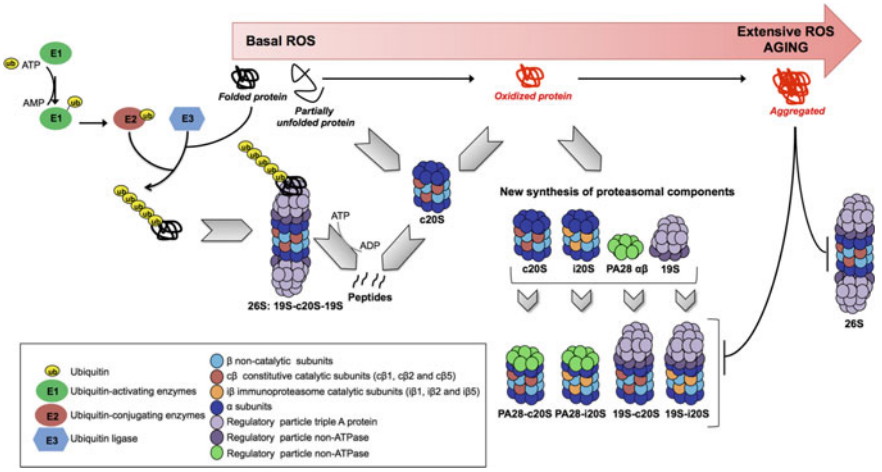

Fig. 8.3 Fundamental pathway of protein degradation by proteasomes during basal and oxidative stress conditions. In basal condition the predominant pathway for protein degradation is the UPS which is an ATP-dependent process mediated by a series of enzymes. The first part of this pathway consists of the transfer of the Ub to the active site of the E2 Ub-conjugating enzymes by the Ub-activating enzyme E1. Then the Ub is ligated by the E3 Ub-ligases to an internal lysine of the target protein. The polyubiquitinated protein is then targeted to the 26S proteasome for degradation. The 26S proteasome is composed of the 20S core particle and of 1 or 2 19S regulatory complexes that bind one or both ends of the 20S. In the early phase of the cellular response to oxidative stress, various changes occur to modulate proteasome activities to promote the degradation of oxidized proteins and to limit stress-related damages. In a first step, under moderate stress conditions, the 26S proteasome is activated. When oxidative stress persists or after the application of acute stress, disassembly of proteasome 26S into proteasome c20S and regulator 19S is observed. In yeast, the ECM29 protein is mandatory for this disassembly. Following this dissociation, the free 20S particles are activated and the oxidized proteins are degraded independently of ATP and ubiquitin. If cells undergo prolonged exposure to oxidative stress (at least 12 h) they will enter the late phase of response to oxidative stress. Although the exact mechanisms are not completely understood, an inhibition of the 26S proteasome by protein aggregates is observed during aging and this inhibition results in the synthesis of novel 20S proteasome components and the formation of functional units proteasome in protein degradation. Interestingly, the constitutive 20S proteasomes and immunoproteasomes are more effective than the standard 26S proteasome to degrade oxidized proteins

Saccharomyces cerevisiae and mammals, in seven different α subunits and seven different β subunits (Fig. 8.3) (Groll et al. 1997).

The proteolysis takes place in the internal catalytic chamber of the proteasome formed by the two β rings. In Archaea, catalytic sites are carried by all β-subunits whereas only three of the seven β-subunits in eukaryotes have a catalytic activity (β1, β2 and β5). The complexity of the catalytic particles goes hand in hand with a specialization of the substrate specificity of these three β-subunits. In fact, Archae proteasomes have a single chymotrypsin activity whereas eukaryotic proteasomes possess three types of catalytic activities: chymotrypsin-like (cleavage after hydrophobic residue), trypsin-like (cleavage after basic residue), and caspase-like or

post-acidic (cleavage after acidic residue). The complexity of the catalytic subunits seems necessary to achieve improved and more specialized proteins degradation. Indeed, the cleavage preferences of β1, β2 and β5 and the resulting peptide products are very different.

Thus, in addition to the three catalytic activities, other types of catalytic particles carrying specialized functions are found in vertebrates (Fig. 8.3). These proteasomes are not ubiquitous as the constitutive proteasome (c20S) since they are found only in specific tissues. They are different from c20S in the composition of their active sites while keeping the same types of activities. The difference lies in the recognition specificity and the nature of the hydrolysis products. These proteasomes are: the immunoproteasome (i20S) and the thymoproteasome (t20S). These specialized proteasomes can perform functions that the constitutive proteasome cannot perform. In general, depending on the nature of the peptide products, the functions are diverse. For example, they can serve for the immune response, in the selection of T lymphocytes (t20S) or to other aspects of cell biology.

The degradation specifications do not rely only on the 20S particle and its three different activities. Indeed, this 20S particle by associating with different regulators can recognize and degrade a large variety of substrates through different pathways. Depending on the type of substrates and on cellular and extracellular conditions, different ways are preferred.

8.3.1.2 The ATP-Dependent Pathways: Ubiquitin Proteasome System (UPS)

The UPS pathway is a major protein degradation system (Fig. 8.3). UPS is present in the cytoplasm and nucleus of eukaryotic cells and is mostly responsible for the intracellular proteolysis. The ubiquitylation cascade involving the enzymes E1, E2 and E3 successively labels proteins by polyubiquitylation which are then directed towards proteolytic degradation by the 26S proteasome that results from the association of two regulatory particles (19S) and the catalytic particle (20S): 19S-20S-19S (Fig. 8.3). Ubiquitin binds by isopeptidic links between the lysine 48 of an ubiquitin and the glycine 76 of the following ubiquitin to form a polyubiquitin chain attached to the protein. The UPS pathway involving the 26S proteasome is a selective ATP-dependent degradation mechanism. Indeed, it ensures the renewal of all types of proteins and in particular regulatory proteins that are generally short-lived. The UPS also allows the elimination of proteins, the accumulation of which could be deleterious for the cell: misfolded or damaged proteins as well as mutant proteins presenting an abnormal conformation. However, other forms of proteasome preferentially provide this function. Alterations of the UPS pathway have been associated with a variety of pathologies which makes it a potential therapeutic target of great interest for the treatment of a number of diseases in humans: cardiovascular diseases, viral pathologies, neurodegenerative disorders and also cancers.

8.3.1.3 The ATP-Independent Pathway

There are other pathways of degradation that do not require protein labeling by ubiquitin and are therefore independent of ATP. These pathways are often activated in response to stress or upon the presence of a pathogen.

One of the most important functions of the immunoproteasome is its role in immunity. Indeed, it allows the presentation of antigens by MHC-1 on the surface of CD8 + T lymphocytes. The constitutive proteasome can also produce antigenic peptides but with different lengths from those generated by the immunoproteasome. It has been proposed that the peptides produced by the immunoproteasome are optimized for the antigen presentation by MHC-1. Indeed, the peptides produced have an average length of 8–10 amino acids. This process is carried out in connection with aminopeptidases (Ferrington and Gregerson 2012). More recent data have expanded the role of the immunoproteasome in controlling key processes of maintaining cellular homeostasis and in responding to a stress. Indeed, its direct involvements in the control of the NF-κB pathway (Maldonado et al. 2013) and in the degradation of oxidized proteins have been demonstrated (Pickering et al. 2010).

Oxidized proteins degradation by the proteasome in basal condition and in oxidative stress situations has been widely studied. These studies converge towards the fact that oxidized protein degradation is carried out preferentially independently of ATP and thus the UPS is not the major way (Davies 2001; Shringarpure et al. 2003). Indeed, the constitutive proteasome c20S is predominantly degrading oxidized proteins and it has been recently shown that the immunoproteasome i20S is also a very important actor as well as the proteasome activator PA28 (Ferrington et al. 2013; Ferrington and Gregerson 2012; Pickering and Davies 2012a; Ethen 2007; Pickering et al. 2010). In fact as described above, the proteasome is a system capable of rearranging the composition of its subunits and its interactions with regulators under oxidative stress conditions. Thus, it efficiently eliminates proteins that have been targeted by oxidative damage, which generally prevents the ubiquitin association with oxidized lysine residues. Indeed, Pickering et al. (2012) have shown that c20S, i20S and PA28αβ, induced during transient stress, were much more effective than the 26S proteasome in degrading oxidized proteins. Moreover, several studies have reported an increase of the 20S proteasome cellular level during oxidative stress resulting from the disassembly of the 26S proteasome into its 20S and 19S components. During this specific condition an enhanced degradation capacity to cope with increasing amount of oxidatively damaged proteins is needed (Grune et al. 2011; Pickering and Davies 2012b).

The proteasome is abundant in cells but it can become limiting for cell survival upon oxidative stress. Hence, the cell responds to oxidative damage by increasing proteasome activities through the rearrangement of catalytic particles and proteasome regulators in order to increase the quantity and the availability of the 20S proteasome and imunoproteasome. The other regulation level occurs by activating the pore opening through interaction of regulators with the 20S to allow the substrate entry. These two levels of regulation can also be controlled by the

transcription of the catalytic subunits and regulatory particles and by post-translational modifications of the catalytic core permitting the pore opening. Interestingly, the circadian clock has recently been shown to intervene in regulating the proteasome at these different levels (Desvergne et al. 2016).

According to Aiken et al. (2011) and as shown in Fig. 8.3 a model for regulating the proteasome during oxidative stress has been proposed (Aiken et al. 2011). This figure shows the possible combinations for the elimination of oxidatively damaged proteins in response to oxidative stress. The c20S is rather resistant to oxidative stress, whereas the 26S proteasome is much more vulnerable, and it can maintain its activity even after treatment with H_2O_2 ranging from moderate to high concentrations. Acute oxidative stress leads to the 26S proteasome disassembly and therefore to the release of the c20S that increases the ability of the cell to degrade oxidized proteins. The reassembly of the 26S proteasome is made possible after oxidative stress allowing the degradation of the ubiquitylated protein substrates. Various proteins appear to be involved in this process. In the mammalian system, the stabilization of the 19S regulator after its dissociation from the 20S proteasome is managed by the chaperone Hsp70 as well as the reassembly of functional 26S proteasomes, once the oxidative stress is finished (Grune et al. 2011). In yeast, the disassembly of 26S proteasomes is dependent on the proteasome-associated protein, Ecm29 and on low level of Hsp90 (Wang et al. 2010).

On prolonged exposure to oxidative stress, proteasome activities are inhibited and a proteasome de novo synthesis signal is emitted. Up-regulation of constitutive and inducible proteasome components leads to the formation of c20S and i20S respectively. These two complexes can associate with the PA28 and/or 19S regulators to form different functional proteasome complexes. In mammals, treatment with proteasome inhibitors can also induce transcription of its subunits genes. Several studies involving *Drosophila melanogaster*, *Caenorhabditis elegans*, and mammalian cells indicate that the transcription factor NRF2 (nuclear factor erythroid derived 2-related factor 2) has a predominant role in the increased expression of c20S, i20S and PA28 proteasome regulator (Pickering et al. 2012; Kwak et al. 2003a, b). NRF2 is the master regulator of antioxidant transcriptional responses, following oxidative stress (Pickering et al. 2012; Zhang et al. 2013). It attaches to the ARE domains present in the promoter of many defense system genes. For example it has been described that the Nrf2 activator sulforaphane increases proteolytic activity by up-regulating the levels of the 20S proteasome catalytic subunits in murine neuroblastoma cells. The induction of proteasome gene expression is also mediated by the related transcription factor, NRF1 (Zhang et al. 2014). Indeed, NRF1 was shown to up-regulate the expression of both 20S and 19S genes in a NRF2-independent manner, in response to proteotoxic stress caused by proteasome inhibition.

It is becoming increasingly clear that the intracellular redox state also regulates proteasome activity. Post-translational modifications can modify protein functions by changing their structures and physico-chemical properties, including biochemical activity, intracellular localization and protein-protein interactions. Various post-translational modifications of the proteasome subunits have been reported such

as phosphorylation, acetylation, myristoylation, ubiquitylation, modification with N-acetyl-glucosamine (O-GlcNAc), S-glutathionylation and oxidation (Sha et al. 2011; Bose et al. 2004; Demasi et al. 2014a). These various modifications result in variable impacts on proteasome function. In general, with oxidations and in particular carbonylation, a loss of proteasome activity is observed (Shamoto-Nagai et al. 2003). However, it was described that carbonylation can also increase proteasome activities depending on the degree of oxidation and the modified subunits. Similarly, reversible S-glutathionylation would act as the triggering of a redox signaling which regulates proteasome activities by the pore opening according to the redox state of the cells (Demasi et al. 2014b). Indeed, several studies have shown that both the 20S proteasome of yeast and human have a rather biphasic response to S-glutathionylation. Indeed, low concentrations of GSH increase the chymotrypsin-like activity while high levels of GSH lead to a decrease in activity (Demasi et al. 2015). Moreover, the ADP-ribosylation of the PI31 kinase regulates its interaction with the proteasome and thus its phosphorylation, which affects the assembly mechanisms of the various subunits (Cho-Park and Steller 2013). Indeed, the assembly of c20S or i20S represents another key mechanism for the regulation of proteasome activities. Interestingly, the disassembly of the 26S proteasome into 19S and 20S particles that enhance 20S proteasomal peptidase activity is promoted by a decreased phosphorylation of proteasome subunits (Bose et al. 2004; Day et al. 2013). Other kinases c-Abl and Arg, tyrosine kinases were reported to phosphorylate the proteasome α4 subunit leading to inhibition of proteasomal peptidase activity (Liu et al. 2006) whearas the polo-like kinase (Plk) increase the 20S proteasome proteolytic activity by phosphorylating the α3 and α4 subunits (Yang Feng and Ferris 2001).

8.3.2 Circadian Regulation of the Proteasome

8.3.2.1 Rhythmicity of Catalytic Subunits Expression and Proteasome Activities

It has been shown in different model organisms that the transcriptional and translational expressions as well as the catalytic activities of proteasome are under a direct or an indirect control of the circadian clock. Indeed, using gene expression profiling Panda et al. (2002) have identified 650 cycling transcripts in the suprachiasmatic nucleus and in the liver of mice and rats (Panda et al. 2002). It is important to note that the transcriptome does not coincide with the proteome since 5–10% of genes assayed are cyclic whereas 20% of soluble proteins are cycling in mouse liver (Reddy et al. 2006; Panda et al. 2002; Ueda et al. 2002; Storch et al. 2002). Interestingly, among the cyclic transcripts many antioxidant enzymes, several enzymes implicated in ubiquitin metabolism and some proteasome catalytic (β1) and regulatory subunits as well as other components of protein quality control such as certain chaperone proteins were found (Panda et al. 2002). Other

experiments conducted by Yan et al. (2008), in different tissues of several mammalian species confirmed the transcriptional control of certain proteasome subunits and antioxidant enzymes (Yan et al. 2008).

Recent studies realized in HEK (human embryonic kidney) 293 cells and HDF (human dermal fibroblasts) synchronized by a serum shock treatment (Balsalobre et al. 1998) provided further evidence that the circadian clock was controlling the transcriptional expression of proteasome subunits as well as its peptidase activities (Desvergne et al. 2016). This finding may have important implications for the rhythmic regulation of the abundance of proteins that are degraded by the proteasome and might also explain previously reported discrepancies between transcriptome and proteome circadian rhythmicity (Luck et al. 2014; Yang and Schmidt 2014). However, the rhythmic pattern observed for the peptidase activities could not be explained by a rhythmic protein expression of the proteasome and immunoproteasome catalytic subunits (β1, β2, β5, iβ1, iβ2 and iβ5). Indeed, the circadian rhythmicity of the transcripts was not observed at the protein level except for iβ5, which indicates that the circadian modulation of proteasome peptidase activities should originate from other mechanisms than circadian de novo synthesis of proteasome catalytic subunits. In addition, the c20S proteasome has a long half-life of 133 h versus 27 h for the 20 S immunoproteasome (Heink et al. 2005). As described above, proteasome catalytic activities can be also modulated by its interaction with regulatory particles. Moreover, in both synchronized HEK 293 cells and HDF the two isoforms of the activator PA28αβ exhibited circadian oscillations at the transcript and protein levels that were matching those observed for proteasome peptidase activities (Desvergne et al. 2016). The fate of the PA28αβ proteasome activator was analyzed since the level of carbonylated protein was initially found to oscillate in a circadian manner, hence suggesting that oxidized proteins degradation is under circadian control. Moreover, it has been shown that the 20S immunoproteasome and PA28αβ do play a key role in oxidized protein degradation.

8.3.2.2 Direct or an Indirect Circadian Regulation of the Proteasome?

The presence of E-Box element has been demonstrated in the promoter of genes of certain antioxidant enzymes and proteasome subunits, but genomic analyses show that only a relatively small number of output genes are directly regulated by core oscillator components (Panda et al. 2002; Yan et al. 2008). Indeed, it has recently been suggested that the circadian clock can govern antioxidant mechanisms by also acting on the protective response to oxidative stress (Stangherlin and Reddy 2013). However, the underlying molecular mechanisms of this clock control and their physiological roles are not completely understood. The NRF2 gene is one of the genes controlled by the circadian clock since it has an E-Box in its promoter and NRF2 expression is impacted upon knockout of the circadian clock bmal1 gene (Fig. 8.4) (Lee et al. 2013). Several studies have also shown that the circadian regulation of NRF2 results in the circadian expression and activation of antioxidant

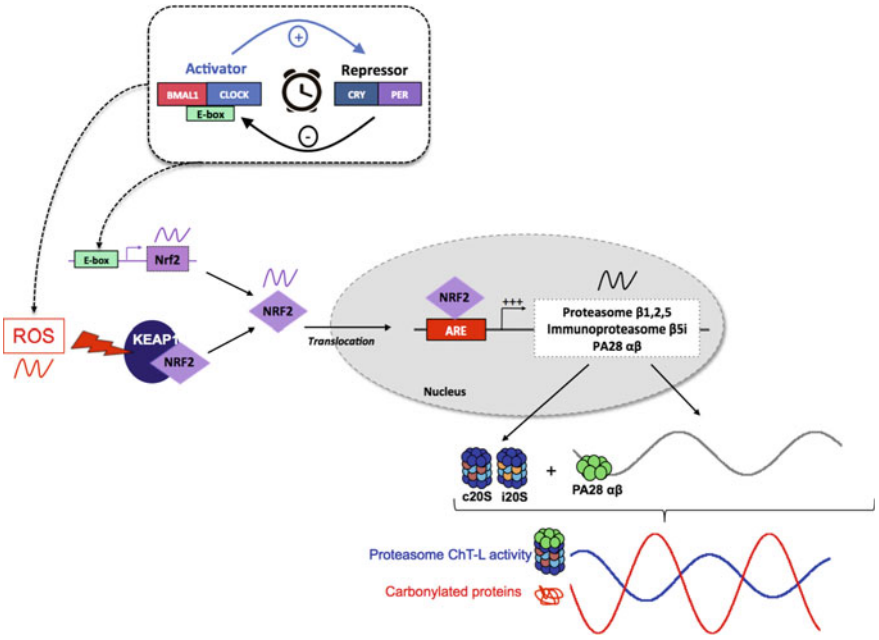

Fig. 8.4 Model for the circadian regulation of proteasome activity and carbonylated protein levels. Reactive oxygen species (ROS) dependent Nrf2-activation, through Keap1 oxidation and further dissociation, has been associated with an increase in the cellular capacity to degrade oxidized proteins that has been attributed to the Nrf2-dependent increased expression of the 20S proteasome subunits and its activator PA28$\alpha\beta$ that contain an ARE motif in their promoter sequence. A circadian expression of Nrf2 was found in synchronized cells while ROS levels are also exhibiting circadian rhythmicity. Hence, the oscillating expression and activation of Nrf2 was found to induce a rhythmic transcriptional synthesis of its target genes, especially the proteasome catalytic subunits *β1*, *β2* and *β5* and immunoproteasome *β5i* as well as the proteasome activator PA28$\alpha\beta$ subunits. The cyclic protein expression observed for the PA28$\alpha\beta$ proteins, unlike the β1, β2, β5 proteasome subunits, argues for PA28$\alpha\beta$ being the main circadian mediated regulator of the rhythmic proteasome activity and rhythmic variation of intracellular protein carbonyl levels

defences, in particular antioxidant enzymes such as those implicated in glutathione metabolism and in the thioredoxin/peroxiredoxin pathway (Xu et al. 2012; Jaramillo and Zhang 2013). In addition, NRF2 stimulates the expression of hundreds genes. Among these genes with AREs in their promoters, several proteasome and immunoproteasome catalytic subunits were found: β1, β2, β5, iβ5 and the proteasome regulator PA28αβ (Fig. 8.4) (Pickering et al. 2012). Interestingly, a circadian expression of NRF2 was observed in both synchronized HEK 293 cells and HDF, that would result in a PA28αβ driven circadian modulation of proteasome activity (Desvergne et al. 2016).

As previously mentioned, the proteasome activities are not only regulated by de novo synthesis of catalytic and regulatory subunits or by activation by regulators, but there is also a control through post-translational modifications. Indeed, the

proteasome is subjected to various modifications such as phosphorylation, acetylation, myristoylation, ubiquitylation, modification with N-acetyl-glucosamine (O-GlcNAc), S-glutathionylation and oxidation. Interestingly, the most important post-translational modifications as phosphorylation and oxidation have been shown to exhibit circadian rhythmicity (Reddy et al. 2006; Desvergne et al. 2016), which could also explain the cyclic activity of the proteasome. Interestingly, Reddy et al., (2006) combined 2D-DIGE with phospho-protein staining and observed out of the 36 rhythmic spots for which phosphorylation state could be determined, 24 (67%) represented phospho-proteins (Reddy et al. 2006). Moreover, S-glutathionylation might also be a cyclic process since the clock via NRF2 regulates phase II enzymes. This process is also observed in basal condition to play an essential role in cell signaling in the same way as reversible cysteine oxidation generated by reactive oxygen species (ROS) . Indeed, ROS can act as important signaling molecules for various cellular functions including circadian rhythms (Brown and Griendling 2009; Tamaru et al. 2013) in many organisms such as Neuropora (Gyongyosi et al. 2013), Drosophila (Grover et al. 2009), zebrafish (Hirayama et al. 2007) and humans (O'Neill and Reddy 2011). Interestingly in flies with a functional clock, daily fluctuations in both the levels of mitochondrial ROS and carbonylated protein, indicative of a circadian rhythmicity in the production of ROS and removal of protein oxidative damage have been demonstrated (Krishnan et al. 2008). Such a circadian rhythmicity of the level of ROS, oxidized glutathione and carbonylated proteins has also been recently reported in synchronized human cellular models HEK 293 and HDF (Desvergne et al. 2016). Hence, proteasome carbonylation could occur in a rhythmic way and thus influence its activity.

8.3.3 Connection Between Proteasome Activities, the Circadian Clock and Aging

A hallmark of aging both at the cellular and organismal level is the accumulation of oxidatively damaged proteins. Protein carbonylation is an irreversible protein oxidation reaction that promotes the accumulation of oxidized proteins and their aggregation. It has been proposed that this accumulation during the aging process may have different origins: increased ROS production, decreased efficacy of the antioxidant defense systems and decreased ability to remove oxidized proteins by the protein maintenance systems such as the proteasome (in the cytosol and the nucleus), the Lon protease (in the mitochondria) and the lysosomes (Hamon et al. 2015; Vanhooren et al. 2015; Chondrogianni et al. 2014). These different possibilities are not mutually exclusive and evidence for either or both hypotheses has been reported depending on the cells, tissues or organisms under study. It seems that the classical enzymes involved in the detoxification of ROS such as superoxide dismutases, catalases, methionine sulfoxide reductases, glutathione transferase and peroxidases are key members of cellular defense and also protect proteins against

carbonylation. In general, the overall activity of these systems has been found to diminish with age. However, in yeast, the increase in protein carbonylation has been linked to an increased tendency of mitochondria to produce ROS during aging rather than a decreased activity and/or abundance of antioxidant systems.

Another possibility relates to the efficacy of the elimination systems, in particular, of the cellular proteolytic systems. A general decline in all these maintenance systems has been associated with aging. The proteasome is involved in many cellular processes and its activity modulations have drastic consequences on normal or pathological processes. We and others have been pioneers in evidencing an age-related decline of proteasome activity and in its involvement in the accumulation of damaged proteins observed during aging (Conconi et al. 1996; Petropoulos et al. 2000; Baraibar and Friguet 2012). Proteasome activity has been shown to decrease both in senescent cells but also in various tissues of aged rats such as heart (Bulteau et al. 2002), spinal cord (Keller et al. 2000), brain (Dasuri et al. 2009), retina and muscle (Ferrington et al. 2005). Even if the mechanisms underlying the age-associated alterations of proteasome function are not completely understood, it has been shown that they are due mainly to a decreased expression and assembly of the proteasome β catalytic subunits, to oxidative damage of the proteasome itself and to the increased formation of protein aggregates that are acting as inhibitors of the proteasome.

As pointed out earlier, circadian rhythm has the ability to generate 24-h periodicities of a large number of biological processes including the production of reactive oxygen species and the regulation of antioxidant defenses (Hardeland et al. 2009). Some of the age-associated changes of circadian rhythmicity described for mammalian organisms and humans are most likely caused by a dysfunction in the organization and activity of the "central pacemaker" or suprachiasmatic nucleus (Hofman and Swaab 2006). However, other organs hosting "peripheral clocks" such as the liver have also shown age-associated variations of the circadian clock genes expression (Claustrat et al. 2005). In addition, circadian rhythmicity is altered in senescent smooth muscle vascular cells due to modified circadian clock genes expression in senescent cells both in vitro and in vivo (Kunieda et al. 2006). Also, the abrogation of the circadian clock in mice for which the circadian clock genes bmal1, per1 and per2 have been inactivated, induces a premature aging phenotype (Kondratov 2007), while the overexpression of the circadian clock period gene leads to increased healthy longevity of *Drosophila melanogaster* (Krishnan et al. 2009). Interestingly, supplementation with the antioxidant N-acetyl-cysteine made it possible to improve the symptoms of premature aging associated with the deficiency of the Bmal1 protein in the mouse (Kondratov et al. 2006).

Recent experiments carried out in our laboratory on senescent dermal fibroblasts, a cellular model of aging, suggest that the decreased proteasome activity observed during aging may be related to the circadian clock perturbation. Indeed, we have observed with this senescent cellular model, an alteration of the circadian rhythmicity characterized by a decrease of the amplitude as well as an increase of the period's length of the clock gene *bmal1* and *per2* expression (Desvergne et al. 2016). Furthermore, in contrast with the synchronized young fibroblasts, neither

proteasome activities, protein carbonyls, NRF2, ROS and oxidized glutathione levels showed circadian rhythmicity in senescent fibroblasts. The link between the biological clock and protein redox homeostasis, including proteasome mediated oxidized protein degradation, could thus explain, at least in part, those changes, described previously and observed during the aging process. Interestingly, the premature aging phenotype observed in bmal1 deficient mice has been linked to an increase of ROS levels compared to wild type mice that was suggested to originate as a consequence of the loss of the transcription activation by CLOCK/BMAL1 of the antioxidant enzymes (Kondratov et al. 2006). In the light of our results, defects in protein redox homeostasis and alteration of proteasome function may also have contributed to this accelerated aging. Taken together, our results support a critical link between the circadian clock, redox and protein homeostasis and the accumulation of oxidized protein during cellular aging.

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Chapter 9
Circadian Clocks and mTOR Signaling

Richa Gupta and Roman V. Kondratov

Abstract The interest in the delay of aging and age associated pathologies through dietary interventions is growing. Understanding the molecular mechanisms of dietary effects on physiology and metabolism is essential to develop rational strategies for dietary interventions. Circadian clock and mTOR signaling pathway play critical role in the organism/diet interaction and regulation of metabolism. Circadian clock generates rhythms in physiology, known as circadian rhythms, which are essential for the synchronization of various metabolic processes with periodic feeding (activity)/fasting (sleep) cycles. Clock disruption is associated with the development of multiple pathologies in animal models and increases the risk of diseases in humans. mTOR signaling pathway is a master regulator of a switch between catabolism and anabolism in response to feeding. mTOR pathway is deregulated in many diseases such as cancer and diabetes and contributes to development of these pathologies. Both systems, the circadian clock and mTOR pathway, have been implicated in the control of aging in different organisms. Recently several groups reported on the existence of the crosstalk between the circadian clock and mTOR signaling pathway. Here we will discuss the role of this crosstalk in aging.

Keywords Circadian clock · Metabolism · Aging · Signal transduction Biological rhythms

9.1 Introduction

The decline in biological activities and impairment of physiological and metabolic functions with age is well known. Aging increases the rate of development for many pathologies such as macular degeneration, atherosclerosis, heart failure,

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neurodegeneration and cancer. The hallmarks of aging include changes in genomic instability, telomerase activity, epigenetic alterations, loss of proteostasis, defects in nutrient sensing, mitochondrial dysfunctions and cellular senescence (López-Otín and Kroemer 2013; Kondratov and Antoch 2006). For a long time diets and nutrients were considered as important effectors of health and disease. Various dietary interventions have been proposed and/or advertised in mass media as methods to improve health and longevity. Some of these diets, such as calorie restriction, have been used in experimental systems in order to increase lifespan and delay aging, while others, such as high fat diet, have been used to induce experimental pathology such as metabolic syndromes. Several physiological systems are involved in the organism's response to the diet and mediate beneficial or negative effects of the diet. One of these systems is the circadian system or circadian clock.

Circadian clocks generate biological rhythms with period of about 24 h. Circadian clocks have been reported for many organisms from unicellular to mammals. It was postulated that the circadian rhythms are essential for the organism/environment interaction and for the synchronization of various physiological processes in the organism. Role of the circadian clocks and rhythms in physiology have been demonstrated in experimental systems; animals with circadian clock disruption through mutation or deletion of clock genes demonstrate disrupted rhythms in behavior and physiology and develop variety of pathologies such as metabolic syndromes, cancer, premature aging, cardiovascular and renal abnormalities. The importance of circadian clock to human health has been confirmed in epidemiological studies: the shift work-induced dysfunction of the circadian clock increases the risk of cardiovascular diseases, cancer and metabolic syndromes (Green et al. 2008a; Davis et al. 2001; Fujino et al. 2006). Diets have strong effect on the circadian clocks: phases of the clock generated rhythms can be reset according to the time of feeding, high fat diet reduces and calorie restriction increases the amplitude of the circadian rhythms. It was proposed that positive or negative effects of diet on physiology might occur through the effect on the circadian clocks.

Another cellular system essential for the organism/diet interaction is mTOR (mechanistic target of rapamycin) signaling pathway. mTOR signaling pathway is nutrient sensing pathway that has an important role in regulating cell growth and proliferation. Under condition of nutrient availability, mTOR signaling is induced and promotes anabolic processes, such as protein and lipid biosynthesis, and inhibits catabolic processes such as autophagy. Activity of mTOR signaling pathway in major metabolic organs such as the liver, muscle, and adipose tissue plays an important role. mTOR also plays role in regulation of whole body energy homeostasis. mTOR signaling pathway is deregulated in human diseases such as cancer, diabetes and obesity (Laplante and Sabatini 2012).

Both the circadian clock and mTOR pathways are important regulators of aging. Recently several groups have reported interaction between the circadian clock and mTOR signaling in mammals (Cao et al. 2008, 2010, 2011, 2013; Khapre et al. 2014a, b; He et al. 2016; Zhang et al. 2014; de Baaij et al. 2015; Feeney et al. 2016; Lipton et al. 2015; Cornu 2015; Drägert et al. 2015). In this review, we will discuss reciprocal regulation of the clock and mTOR pathway, in connection with their roles in nutrient response and aging.

9.2 Circadian Clocks

Circadian clock is an internal timekeeping system that generates 24 h rhythms in behavior, physiology and metabolism in wide range of organisms. These endogenous circadian rhythms are entrained by 24-h light/dark cycle, which is due to Earth's rotation (Bell-Pedersen et al. 2005). Circadian clock synchronizes multiple metabolic processes within the organism with the environment, which might provide some survival advantage to organism (Langmesser and Albrecht 2006; Edery 2000; Khapre et al. 2010). There are many excellent reviews on the circadian rhythms and details on circadian clock organization and functions in different organisms can be found there, however in this review we will briefly discuss the circadian clock and its physiological function in mammals including humans.

Similar to other organisms the human physiology is subject to strong circadian control, daily rhythms are observed in a variety of biological processes, such as gene expression, body temperature changes, sleep/wake cycle, heart rate and melatonin production (Schmidt et al. 2007). The anatomical and physiological clock organization is presented in Fig. 9.1. The master or central circadian clock is located in SCN of the anterior hypothalamus (Reppert and Weaver 2002). Photic stimuli received by retina are transmitted to SCN via retinohypothalamic tract. Synchronization of SCN neurons by daily light/dark cycle generates rhythms in humoral and electrical signals. In addition to central clock located in brain, mammals have similar clocks found in peripheral tissues, such as the liver, intestine and adipose tissue, called peripheral clocks. SCN communicates by means of humoral and neural signals with the peripheral clocks, which produce rhythmic physiological outputs in peripheral tissues, necessary to assure healthy physiology (Green et al. 2008b) (Fig. 9.1). Many non-photic factors such as feeding time, exercise schedule, social interaction and sleep/wake schedule can also synchronize clocks. Circadian clocks operate in almost all the cells of organisms and circadian rhythms generated are governed by the expression of certain set of genes called the core circadian clock genes. These core clock genes and their products (illustrated in Fig. 9.2) form the transcriptional-translational feedback loops (TTFL) and generate circadian rhythms in gene expression in both SCN and peripheral tissues (Lowrey and Takahashi 2004). Transcriptional factors Circadian locomotor output cycles kaput (CLOCK), its paralog Neuronal PAS domain-containing protein 2 (NPAS2) and Brain and muscle aryl hydrocarbon receptor nuclear translocator-like protein 1

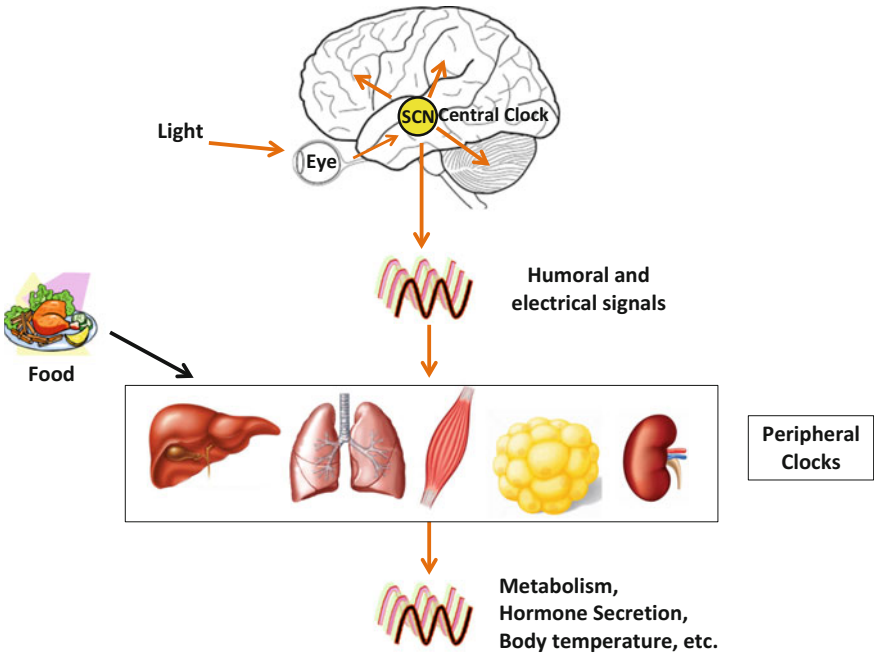

Fig. 9.1 Circadian clocks. The central or master circadian clock resides in the SCN of the hypothalamus and is entrained by the light through retina. The central clock produces signals that synchronize peripheral clocks located in different tissues such as the liver or muscles. The peripheral clocks generate 24-h rhythms in gene expression and multiple physiological functions such as metabolism, sleep/wake cycle, body temperature, hormone secretion etc. Other environmental cues such as feeding can also regulate the peripheral clocks

(BMAL1) are known as positive elements of the TTFL. BMAL1 and CLOCK or NPAS2 form complex and transcribe following genes: *Periods* (*Per1*, *Per2* and *Per3*), *Cryptochromes* (*Cry1* and *Cry2*), the nuclear receptors *Rev-Erbα* and *Rev-Erbβ*, which are known as negative elements and the retinoid-related orphan receptors (*RORs*), work as positive element of TTFL. Two different positive-negative feedback loops are formed by these elements. PER and CRY proteins form complexes in the cytoplasm and translocate back to the nucleus, where they bind to the BMAL1-CLOCK complex and suppress its transcriptional activity, thereby inhibiting their own expression. The second feedback loop is formed by REVs and RORs. REVs negatively regulate the expression of *Bmal1* by directly binding to the ROR element in its promoter, whereas RORs positively regulate *Bmal1* expression (Ko and Takahashi 2006). In addition to TTFL other mechanisms regulate the core circadian clock gene expression, such as post-translational and post-transcriptional regulation, also exist (Harms et al. 2004).

9.3 Circadian Clocks and Aging

The circadian clocks are located throughout the body and influence nearly all aspects of physiology and behavior, which helps in regulating rhythmic processes in metabolism. The disruption of the circadian clock has been associated with many age-related phenotypes suggesting that the circadian clock may be able to impede the aging process. Moreover, the disruption of circadian rhythms can seriously impact overall health (Abbott et al. 2015; Ferrell and Chiang 2015; Videnovic and Zee 2015). Importantly, robust circadian rhythms could lead to better health and probably increased longevity. Reduced lifespan and increased rate of age-associated diseases have been reported for several animal models of clock disruption in mice and flies (Krishnan et al. 2009, 2012; Antoch et al. 2008). Mice deficient in *Bmal1* demonstrate a severe accelerated aging phenotype and have dramatically reduced lifespan (Kondratov and Antoch 2006), which suggests that some of the effects of the circadian clock on the aging process are gene dependent. Importantly, longevity extending intervention, the calorie restriction, requires a functional circadian clock for its full benefits. Indeed, calorie restriction cannot increase lifespan of flies with clock disruption (Katewa et al. 2016) or mice deficient for BMAL1 (Patel et al. 2016).

There are multiple recent reviews on the molecular links between the circadian clock and signaling pathways known as hallmarks of aging (see also Fig. 9.3). Circadian clock proteins are implicated in the control of cell death, proliferation, cell cycle control, DNA repair and stress response (Khapre et al. 2010; Kondratov and Antoch 2007b; Sancar et al. 2010; Sahar and Sassone-Corsi 2009; Marcheva et al. 2013). The expression of many cell cycle regulating genes such as cyclin-dependent kinase inhibitors p21, p27, Cdk2, cyclins A, B1, D1, D3, and E1, cyclin-dependent kinases Cdk2 and Cdc2, transcription factors c-myc, Wee1 kinase demonstrate circadian rhythms. Direct interaction was also reported: PER1 (PERIOD1) and TIM (TIMELESS) interact and regulate checkpoint associated protein kinases: ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR) (Kondratov and Antoch 2007a). TIM also interacts with components of DNA replication machinery (Gotter and Emanuel 2007). CLOCK deficient mice do not display rhythmic telomerase activity and their chromosomes have shorter telomeres, thus the interplay between the circadian clock and telomeres exist (Chen and Wang 2014). The expression of apoptosis-related genes, such as p53, mdm2, Apaf1, Dapk1, p63, and Bcl2 is rhythmic and altered in the circadian clock mutants (Khapre et al. 2010). Oxidative stress response and ROS homeostasis is also under circadian clock control. NAD^+-dependent deacetylases known as Sirtuins are important regulators of aging and they are tightly interlinked with the clock. Direct interaction of SIRT6 with BMAL1, CLOCK and PER2 was reported (Fonseca Costa and Ripperger 2015). Sirtuins modify and affect the activity of clock proteins; in turn the circadian system regulates activity of Sirtuins through control of cellular redox state (NAD+/NADH ratio). In agreement with that mutual regulation, the activity of sirtuins is reduced in circadian clock mutants, while age

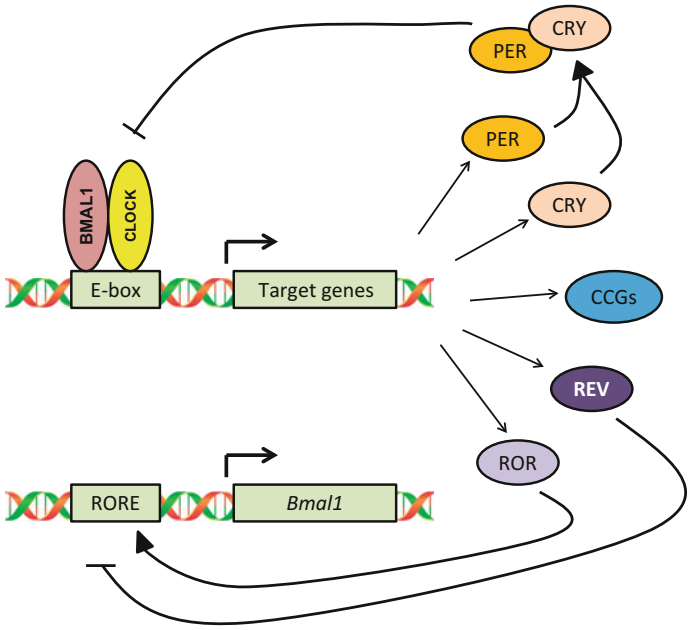

Fig. 9.2 Circadian clock transcriptional-translational feedback loops. On cellular level the circadian clock is organized as interlocking feedback loops formed by several core-clock genes and their products. Transcriptional factors BMAL1 and CLOCK form the complex and drive the expression of other clock genes three periods, two cryptochromes, Rev-Erv alpha and beta and ROR alpha, beta gamma. PER and CRY protein form the complex in cytoplasm and translocate to nucleus, where they inhibit the BMAL1/CLOCK complex activity and their own expression, therefore, forming first negative feedback loop. Revs and RORs regulate Bmal1 transcription, where Revs are negative regulators and RORs are positive regulators of Bmal1 transcription. Thus, Revs, RORs and Bmal1 form second negative-positive feedback loop

dependent changes in SIRT1 activity affects the circadian clock function in the brain (Asher and Schibler 2008; Nakahata and Sassone-Corsi 2008). Cellular senescence and stem cell depletion contribute to the impaired tissue ability to regenerate and circadian disruption affect both (Brown 2014).

Disruption of the control of biological macromolecules and particularly protein homeostasis (proteostasis) is an important contributor to aging process. Circadian regulation of protein translation and degradation (through ubiquitin/proteasome and through autophagy) has been reported. mTOR signaling pathway is a master regulator of protein anabolism and catabolism. It which is also involved in aging. We will focus below on recently reported reciprocal interaction between mTOR and the circadian clocks.

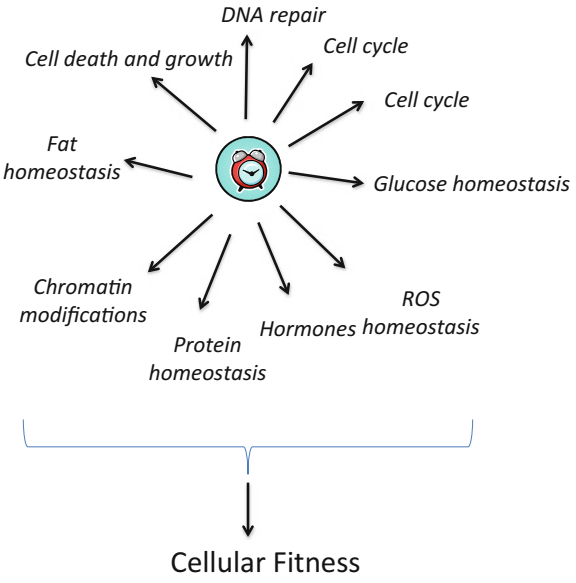

Fig. 9.3 Circadian clock and hallmarks of aging signaling pathways. Circadian clocks regulate cell metabolism, signaling and multiple cellular processes such as DNA repair and ROS homeostasis. All these processes are important for cellular physiology and synchronization of the cell with tissue and organism. Clock disruption affects these processes and will compromise cellular fitness

9.4 mTOR Signaling Pathway

There are multiple reviews on mTOR signaling and its role in nutrient response and aging in different organisms, details can be found there (Johnson et al. 2015; Zoncu et al. 2011), here we provide only short overview of mTOR pathway in mammals. The target of rapamycin (TOR) is a conserved serine/threonine kinase that belongs to the phosphatidylinositol 3-kinase (PI3K)-related kinase (PIKK) family. TOR was discovered by the isolation of rapamycin resistant mutants of budding yeast Saccharomyces cerevisiae. Rapamycin (also known as Sirolimus), an anti-fungal, immunosuppressant and anti-cancer compound was first produced by the bacterium Streptomyces hygroscopicus in 1972 (Heitman et al. 1991; Cafferkey et al. 1993). TOR exists in two structurally and functionally distinct complexes: TOR complex 1 (TORC1 in yeast and mTORC1 in mammals) and TOR complex 2 (TORC2 in yeast and mTORC2 in mammals). The compositions of TORC1 and TORC2 complexes are conserved from yeast to mammals. Figure 9.4 illustrates the composition of mTOR complexes in mammals. Three proteins are common components of both complexes: the TOR kinase, the DEP domain containing mTOR-interacting protein (DEPTOR) and the mammalian lethal with Sec13 protein 8 (mLST8). DEPTOR acts as a negative regulator for both TORC1 and TORC2 (Peterson et al. 2009) and mLST8 has unknown function but required for the activity of mTORC2 (Guertin et al. 2006). Other components of mTORC1 include the regulatory-associated protein of mTOR (RAPTOR) and the proline-rich Akt substrate of 40 kDa (PRAS40). RAPTOR is essential for kinase activity of mTORC1 and PRAS40 is a negative regulator of mTORC1. mTORC2 unique

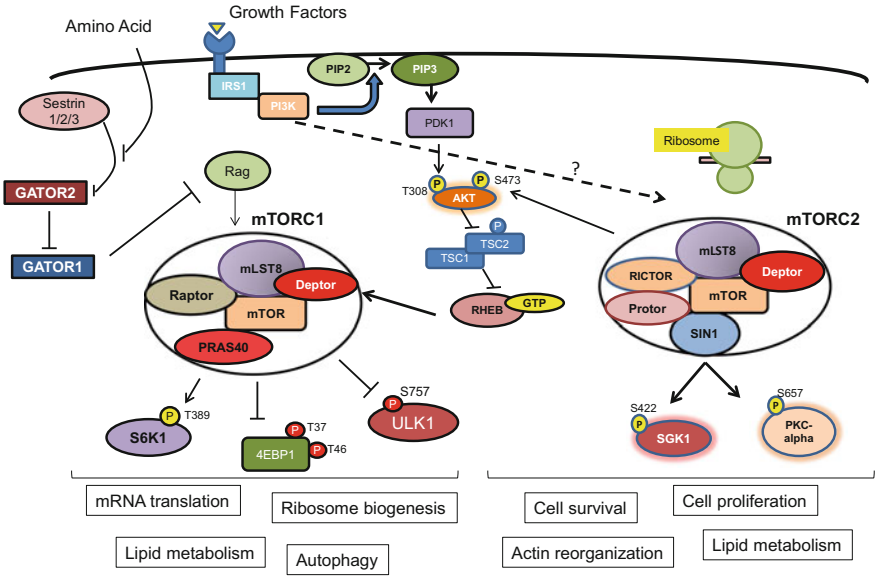

Fig. 9.4 mTOR signaling. Two distinct mTOR complexes are regulated through different but partially overlapping pathways and different downstream targets and cellular processes. mTORC1 is activated by growth factors and nutrients: one major pathway is a cascade of phosphorylation from growth factor receptors through PI3K and AKT to TSC complex, which regulates GTPase Rheb. Another pathway is induced by amino acids availability and involves Gators complexes, which regulates GTPase Rag. Active mTORC1 will phosphorylate downstream targets such S6K1, 4E-BP and ULK1 to stimulate anabolic and suppress catabolic processes. mTORC2 is activated in response to insulin through PI3K dependent mechanism but exact pathway is not well characterized, an association with ribosome is also essential for activation. mTORC2 phosphorylate its targets such as AKT and SGK in order to regulate cell survival, actin reorganization, glucose and lipid metabolism

components are the rapamycin-insensitive companion of mTOR (RICTOR), protein observed with RICTOR (PROTOR), mammalian stress-activated protein kinase-interacting protein 1 (mSIN1), and proline-rich protein 5 (PRR5). RICTOR and SIN1 regulate the assembly of mTORC2 complex and are essential for its kinase activity (Chen and Sarbassov 2011). PROTOR is not required for binding of RICTOR and SIN1 to the complex (Pearce et al. 2007) but affects mTORC2 dependent phosphorylation of some down-stream targets. Protor deficiency does not affect phosphorylation of AKT and PKC-alpha on mTORC2 dependent sites but SGK1 phosphorylation is reduced at least in some tissues (Pearce et al. 2011). mTOR complexes differ in their sensitivity to rapamycin. mTORC1 is acutely sensitive to the rapamycin treatment whereas mTORC2 is affected only after long exposure (>24 h) to rapamycin (Wullschleger et al. 2006; Sarbassov et al. 2006).

mTOR complexes have some common and some different upstream regulators, downstream targets are also different, which determine different biological roles of the complexes. mTORC1 senses many extracellular cues such as nutrients

(e.g. amino acids), growth factors (e.g. insulin, IGF1) and stress to coordinately regulate cellular responses to these environmental signals (Wullschleger et al. 2006; Laplante and Sabatini 2012). mTORC1 is activated by two parallel pathways: (1) by growth factors (insulin, IGF-1, PDGF), through TSC/Rheb dependent mechanism. Rheb is a small GTPase, which activity is negatively regulated by GTPase-activating protein (GAP) TSC1/TSC2, which converts Rheb into its inactive GDP-bound state. This inhibitory function of TSC1/TSC2 complex is attenuated by active PI3K-AKT pathway in response to growth factors (Laplante and Sabatini 2009a). (2) By the amino acids through Rag dependent mechanism. Rags are small GTP binding proteins, their activity is negatively regulated by GTPase-activating protein complex called GATOR1 (GTPase-activating proteins toward Rags 1) complex. GATOR1 complex activity is inhibited by GATOR2 complex through direct interaction between the complexes. Sestrins, stress-inducible metabolic regulators, inhibit mTORC1 activity by interacting with GATOR2, which releases GATOR2 from GATOR1 complex and therefore, GATOR1 can inhibit Rags (Bar-Peled et al. 2013). Branched amino acids, such as leucine, inhibit interaction between sestrins and GATOR2 complex (Kim et al. 2015; Wolfson et al. 2016).

TORC1 regulates several cellular processes: mRNA translation, ribosome biogenesis, lipid metabolism and autophagy. mTORC1 regulates global mRNA translation and ribosome biogenesis by phosphorylating 4EBP1 and p70 ribosomal protein S6 kinase 1 (S6K1) (Thoreen et al. 2012; Hsieh et al. 2012; Mahoney et al. 2009). mTORC1 positively regulates lipid biosynthesis mainly through phosphorylation of sterol regulatory element-binding protein-1c (SREBP-1c), a master transcription factor regulating gene required for lipids biosynthesis (Laplante and Sabatini 2009b). Under nutrient rich condition mTORC1 is active and inhibits autophagy by phosphorylating ULK1 in mammals (Atg13 in yeast), which is an initiator of autophagy and its phosphorylation inhibits the initial autophagy complex formation (Mizushima 2010).

mTORC2 is mainly activated by growth factors such as insulin (Oh and Jacinto 2011). Amino acid can also regulate mTORC2 activity, depending on substrates and cellular context (Yan et al. 2012a, b; Buchan and Parker 2009). Although molecular mechanism of mTORC2 activation is not clear but its association with ribosome is necessary for its activation in PI3K dependent fashion (Zinzalla et al. 2011). mTORC2 regulates variety of cellular processes such as cell survival, proliferation and actin reorganization. mTORC2 phosphorylates AKT at Ser473 within the hydrophobic motif, which is required for its full activation (Sarbassov et al. 2005). mTORC2 also phosphorylates AKT at Thr450, this phosphorylation occurs even in absence of growth factors (Facchinetti et al. 2008). mTORC2 regulates sodium transport and cell survival by phosphorylation and activation of SGK1 at Ser422. (Lu et al. 2011). mTORC2 phosphorylates PKC-alpha at Ser657 to activate RhoA and Rac1 and regulate actin reorganization (Laplante and Sabatini 2012; Oh and Jacinto 2011; Malik et al. 2013). Recent studies have also shown that mTORC2 positively regulates lipogenesis, through AKT-mediated activation of SREBP-1c (Hagiwara et al. 2012).

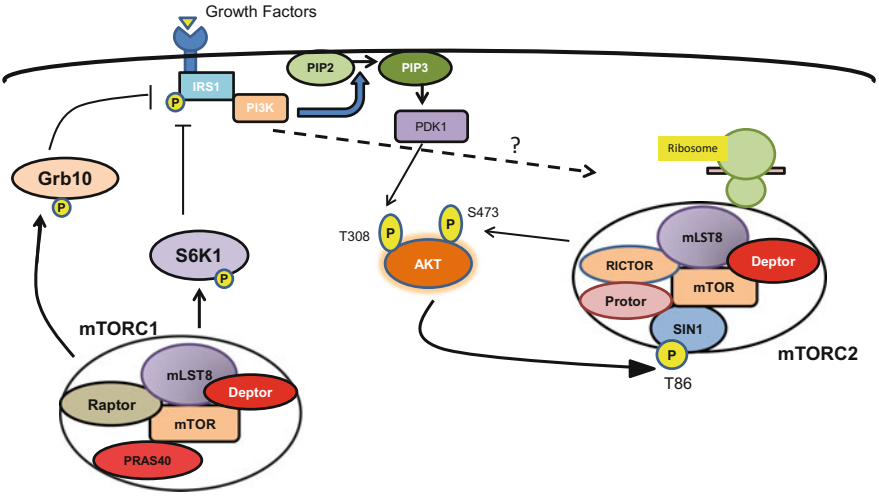

Fig. 9.5 Crosstalk between mTOR complexes. mTORC1 phosphorylates its substrates: Grb10 and S6K1, this phosphorylation of Grb10 and S6K1 leads to suppression of PI3K signaling pathway through inhibitory phosphorylation of IRS1. PI3K is upstream regulator of both mTOR complexes and its suppression leads to inhibition of mTOR signaling pathway (negative feedback loop). There is positive feedback loop between mTORC2 and AKT. PDK1 partially activate AKT, which phosphorylate SIN1 at T86. SIN1 phosphorylation activates mTORC2 which leads to AKT phosphorylation at Ser473

In addition to differential regulation there is a crosstalk between complexes (Fig. 9.5). Chronic activation of mTORC1 leads to inhibitory phosphorylation of IRS1 by mTORC1 and by its downstream target S6K1 (Um et al. 2004). This phosphorylation causes dissociation of IRS1 from InsR and IGFR resulting in diminished activation of PI3K. Another substrate of mTORC1, Grb10, also acts as a negative regulator of insulin/IGF signaling by inhibiting PI3K (Yu et al. 2011). Because PI3K is upstream for both complexes, mTORC1 down-regulates its own activity (negative feedback loop) and mTORC2 activity. mTORC2 dependent phosphorylation of AKT at Ser473 increases the kinase activity and might generate a positive feedback loop for the mTOR complexes (Yang et al. 2015).

9.5 mTOR Signaling and Aging

The importance of TOR signaling in aging has been established over the last decade. Originally it was found in yeast that genetic ablation of some of the pathway components increases yeast lifespan. Later it was confirmed in other model organism such as worms and flies. Finally, pharmacological inhibition of TORC1 signaling extends lifespan in mice, suggesting universal role of mTORC1 signaling in aging (Powers et al. 2006). Lifespan extending paradigm such as calorie

restriction also affects TOR signaling, in worms and flies the activity of mTORC1 is reduced under calorie restricted conditions (Kaeberlein et al. 2005) but in mammals the existing reports are contradictory (Mattison et al. 2012; Colman et al. 2014). Inhibition of mTORC1 also delays development of age-related diseases, suggesting that mTOR signaling may provide better insight on aging and age-related disease and understanding of mTOR signaling help to develop strategy to treat age-related diseases.

It was proposed that deregulated mTORC1 pathway is a major driver of aging (Blagosklonny 2011), how does mTORC1 promote aging? *Tor* deficiency is embryonically lethal in mice, which limits ability for genetic analysis. At the same time, knockouts for some mTORC1 downstream targets have been generated. S6K1 is major downstream target and effector of mTORC1 signaling, S6K1 knockout mice live significantly longer than wild type mice and display a phenotype similar to the phenotype of dietary restricted mice. S6K inhibition also extends lifespan in yeast, worms and flies (Kaeberlein et al. 2005; Kapahi et al. 2004). Furthermore, the overexpression of a constitutively active form of S6K in D. melanogaster renders flies resistant to lifespan extension upon rapamycin treatment (Selman et al. 2009; Fabrizio et al. 2001). 4E-BP is the other well-characterized downstream target of TORC1, mTORC1 dependent phosphorylation triggers 4E-BP degradation. 4E-BP regulates the expression of stress responsive genes such as FoxO and Nrf, whose activity exert a positive effect on lifespan by protecting cells and tissues from age-related damage. Hence, TORC1 may control aging by suppressing activation of stress responsive genes downstream of 4E-BP. Other possibility is mTOR1 dependent regulation of autophagy. Active mTORC1 suppress autophagy through the phosphorylation of rate limiting components of autophagosome formation. Reduced autophagy was linked with aging and in agreement with that mice with brain specific deficiency for atg-7 or atg-5 shown shorter lifespan and accelerated aging (Toth et al. 2008; Komatsu et al. 2006; Hara et al. 2006). In summary, it appears that reducing TORC1 signaling specifically in a metabolic tissue may be sufficient to extend lifespan or delay age related pathologies (Polak et al. 2008). All these data together support evolutionary conserved role of mTORC1 in aging and make the component of the pathway attractive candidates for anti-aging interference. However, it is also important to mention that in mammals the effect of mTOR suppression might be tissue specific and not necessary beneficial. Mice with skeletal muscle specific *Tor* deficiency develop progressive muscle dystrophy and display decreased oxidative capacity and increased glycogen content (Yecies et al. 2011; Risson et al. 2009). The cardiac-specific *Tor* or *raptor* knockout mice develop dilated cardiomyopathy.

The role of mTORC2 in aging is less clear. Recently it was shown that the deletion of *Rictor* (major component of mTORC2) results in decreased lifespan of male mice but has no effect on female mice lifespan. The role of TORC2 in *C. elegans* is complex: worms with RNAi knockdown of rictor at adulthood have increased lifespan (Robida-Stubbs et al. 2012), whereas *rictor* null mutant shows diet dependent effects on longevity. *Rictor* null mutant shows decreased lifespan on the standard laboratory diet and increased lifespan on the nutrient rich diet. Thus,

the effect of mTORC1 and mTORC2 inhibition has different effects on aging in *C. elegans* at the standard diet conditions: low mTORC2 activity is associated with the decrease in lifespan and low mTORC1 activity is associated with increased lifespan (Soukas et al. 2009).

mTORC1 and mTORC2 have different effect on glucose and fat metabolism. Mice with the adipose tissue-specific deletion of *raptor* (reduced mTORC1 activity) are lean and protected against diet-induced obesity (Polak et al. 2008). Decreased mTORC1 signaling leads to the reduced expression of the adipogenic transcription factors such as peroxisome proliferators-activated receptor gamma (PPAR gamma) and CCAAT/enhancer binding proteins, thus interfering with adipocyte differentiation program and homeostasis (Kim and Chen 2004). On the contrary, for adipose tissue-specific rictor knockout mice (reduced mTORC2 activity) the fat mass is largely unaffected. Mice with liver specific ablation of mTORC2 have systemic hyperglycemia, hyperinsulinemia, and hypolipidemia (Hagiwara et al. 2012). An increase in hepatic mTORC1 activity by liver-specific knock out for *Tsc* results in hepatic and systemic insulin resistance, increased fat utilization and glucose production (Kenerson et al. 2011).

9.6 Circadian Clock and mTOR Signaling

The central circadian clock or SCN clock located in hypothalamus is regulated by light signals received by retina. Light signals transduced to SCN are known to reset clock by regulating transcription and translation of core clock genes. It was demonstrated that activity of mTORC1 in the SCN is regulated by photic signals in phase dependent manner. Light signals during dark phase induce phosphorylation of mTOR downstream targets, S6K1 and 4EBP1 in the SCN (Cao et al. 2008). It was further shown that light induced mTORC1 activation results in increased PER1 and PER2 protein expression in the SCN and inhibition of mTORC1 with rapamycin causes defect in light induced phase shifting in mouse locomotor activity. These data indicated that mTOR pathway is a potent light induced regulator of SCN clock entrainment (Cao et al. 2010). The mTORC1 activity in the SCN shows circadian rhythms that correlate with the rhythms of the clock gene *Per1* expression. Together these data indicate that mTORC1 activity across the day is controlled by SCN clock (Cao et al. 2011). SCN clock also controls the circadian rhythms of 4EBP1 expression in mTORC1 dependent manner. Physiological importance of these regulation has been demonstrated using 4E-BP1 null mice, these mice demonstrate faster re-entrainment of upon light/dark cycle shift and they are resistant to constant light induced clock desynchronization. Authors of these studies proposed that translational control by mTORC1/4EBP1 is necessary for entrainment of SCN clock (Cao et al. 2013).

Peripheral clocks are also connected with the mTOR signaling. Activity of mTORC1 in the liver and some other peripheral organs demonstrates circadian rhythms under both feeding and fasting conditions, thus, these rhythms are not

simple consequence of periodic feeding and some clock is involved in the generation of these rhythms. However, these rhythms in mTORC1 activity were preserved in mice deficient for the core clock genes *Bmal1* and *Cry1,2* (Khapre et al. 2014a), which suggests that they are regulated by some clock, which is not identical with the light entrained circadian clock, this hypothetical clock was named Nutrient Anticipation Metabolic Oscillator (NAMO). Food entrained oscillator (FEO) is reported example of the circadian clocks that is SCN independent clock; whether rhythms in mTORC1 signaling link with this clock or not is unknown. Transcriptional factor BMAL1 negatively regulates mTORC1 activity in several tissues, the regulation might, at least partially, occur through the transcriptional control of *Deptor* expression. The BMAL1 dependent regulation of mTORC1 activity occurs on cell autonomous level, Bmal1 deficient cells demonstrate increased mTORC1 signaling and cell proliferation. It is possible that regulation of mTORC1 is clock independent function of BMAL1 (Khapre et al. 2014b). Importantly, the increased mTORC1 activity contributes to premature aging phenotype in Bmal1 deficient mice and pharmacological suppression of mTORC1 activity by rapamycin increases lifespan of these mice. In agreement with these data cardiomyocyte-specific Clock or Bmal1 disruption causes an increased activation of mTORC1 signaling (He et al. 2016). BMAL1 also regulates mTORC2-AKT pathway through the control of RICTOR expression (Zhang et al. 2014). Daily fluxes of magnesium that regulates circadian timekeeping and metabolism might represent another mechanism of circadian regulation of mTORC1 activity. Indeed, Mg^{2+} acts as a second messenger to activate protein synthesis via mTORC1 (de Baaij et al. 2015) and reported circadian rhythms in intracellular Mg^{2+} levels might contribute to the circadian translational control (Feeney et al. 2016).

Another important connection between BMAL1 and mTORC1 pathway has been reported by Lipton et al. (2015). BMAL1 forms complex with the components of the cap-binding complex—eukaryotic translation initiation (eIF) and elongation factors (eEFs) and polyadenylate binding protein 1 (PABP1). The complex binds to 7-methyl guanosine (m7-GTP) and form 'cap' like structure at 5′ end of most mRNAs. These cap-binding complexes have been found in mouse liver and brain and shown to stimulate cap-dependent translation in MEFs. S6K1 phosphorylates BMAL1 at Ser42 and this phosphorylation is required for BMAL1 association with the factors involved in cap-dependent translation. S6K1 is one of the mTORC1 targets, thus BMAL1 regulates cap-dependent translation in mTOR-dependent manner. Interestingly, while BMAL1 is a transcriptional factor its ability to regulate translation is independent from its transcriptional activity (Lipton et al. 2015). Figure 9.6 summarizes interaction linking the circadian clock proteins, circadian clocks and mTOR pathway.

Recently role of mTORC2/Rictor in regulation of peripheral and central clock gene expression was also proposed. Mice with reduced mTORC2 in the liver (due to *Rictor* deficiency) do not demonstrate any significant difference in the expression of core circadian clock genes in liver. Rhythms in body temperature and locomotor activity was also not affected in these mice (Cornu 2015). On the other hand, mice

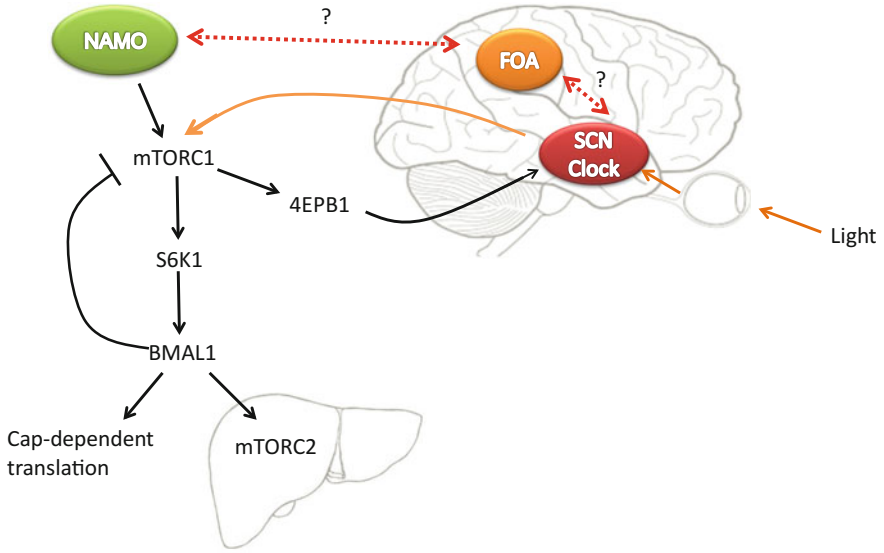

Fig. 9.6 Circadian clock and mTOR signaling pathways. mTORC1 activity in the central clock (SCN) is regulated by the light and this activity through 4E-BP dependent mechanisms contribute to the circadian clock entrainment. Circadian rhythms in mTORC1 signaling exist in both SCN and peripheral tissues, these rhythms can be generated by the light entrained circadian clock and/or by other circadian clocks such as FAO or NAMO. BMAL1 regulates mTORC1 (negatively) and mTORC2 (positively) activity through unknown mechanism. In turn, S6K1, mTORC1 downstream target, phosphorylates BMAL1 and BMAL1 regulates translation

with *Rictor* knockdown in adipocytes show disrupted expression of several core clock genes in adipocytes; interestingly the circadian rhythms of blood pressure and locomotor activity were altered in these mice (Drägert et al. 2015).

9.7 Conclusion

Aging of human population in the developed countries and around the world brings new challenge for biomedical sciences. Delay or prevention of age-associated diseases will have significant social and economic impact. Evidence accumulates that mTOR signaling pathway might be central for aging and manipulation of this pathway may increase longevity in variety of species including mammals. Here we reviewed recent data on interaction between mTOR pathway and circadian clocks. This connection might help to develop rational strategy to manipulate mTOR signaling through minimal invasion intervention that can complement existing pharmacological or dietary approaches. Thus, further investigation of the role of the circadian system in aging and mTOR signaling is well warranted.

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Chapter 10
Aging and the Biological Clock

Michael Judge, James Griffith and Jonathan Arnold

Abstract Aging and the biological clock are linked via metabolism. We explore how different pathways within metabolism link aging and the clock. These linking pathways include those involving: (1) sphingolipid metabolism; (2) the sir2 complex of genes affecting caloric restriction; (3) pathways underlying oxidative stress; (4) the cell cycle; and (5) glucose metabolism. Making these metabolic linkages in *Neurospora crassa* is facilitated by the availability of an entire transcriptional network for the clock. Emerging themes include epistatic interactions between genes leading to effects on the clock and aging. We also present evidence that these five metabolic linkages affecting aging provide feedback to the clock. Metabolism is not only driven by the clock, but metabolism is likely to provide feedback to the clock by a variety of metabolic mechanisms.

Keywords Biological clock · Race tubes · Sphingolipid · Caloric restriction Oxidative stress · Cell cycle

The biological clock and aging are processes that are controlled by overlapping genetic networks, each involving thousands of genes (Chen et al. 2008; Dong et al. 2008; Zahn et al. 2007; Al-Omari et al. 2015). There are thus many opportunities for these two processes to be linked through metabolism. There are compelling examples of how the clock and aging may be linked. One of the oldest examples involves the examination of the viability of fruit flies as the light (L) and dark (D) times experienced are varied away from the natural L/D cycle (Pittendrigh and Minis 1972). The result was a drop in viability and hence longevity of flies when the time of their exposure to L and D deviated from their natural L/D cycle. Thus, Pittendrigh argued that there was a "resonance" between the natural period of circadian rhythms and the longevity of flies. Similar effects of light-at-night have been seen in mice with effects on longevity, incidence of cancer, and disturbances in estrus cycles (Anisimov et al. 2004).

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Another striking example of the linkage of aging and the clock is through the SIRT1 gene product in mammals. The *SIRT1* gene and other genes involved in the Insulin/IGF pathway have been linked to human longevity in a variety of studies (Willcox et al. 2008; Cohen et al. 2004; Kim et al. 2012). The same SIRT1 gene product has been shown to bind several core clock mechanism gene products, such as CLOCK-BMAL1 complex, and thereby deacetylating the oscillator protein, PER2 (Asher et al. 2008). SIRT1 function is required for robust circadian rhythms of several clock mechanism genes (Asher et al. 2008). Thus, through the longevity gene product SIRT1 there is a direct input signal by metabolism into circadian rhythms. One hypothesis about the SIRT1 gene function in mammals is that it mediates the effects of caloric restriction on lifespan.

The linkage of the clock and aging was examined in the model nematode system, *Caenorhabditis elegans*. Major clock mechanism genes were mutated singly and in combination. Survival curves and associated longevity of these mutants were determined. Some of these mutants (*clk-1*, *clk-2*, and *clk3*) showed an increase in longevity, and one double mutant (*clk-1*, *clk-3*) displayed higher longevity than the constituent single gene mutants (Lakowski and Hekimi 1996). These clock gene effects operated independently from those that influence dauer formation (dormant life stage) (Kenyon 2011), which was well known to increase lifespan under conditions of stress.

Thus in a variety of organisms the clock and aging process, two distinct timing phenomena, are inter-related. The first two examples may be thought of as lifestyle mediating the interaction of aging and the clock. The last example can be thought of as genetic interactions mediating the linkage of the clock and aging.

What is aging? Aging is usually thought of as a developmental process in which there is a steady and progressive decline of function with chronological age associated with an inability to cope with damage, disease, and stress (Poon and Perls 2008; Jazwinski 2002). A slightly more general definition of aging with an evolutionary flavor is a persistent decline in age-specific "fitness components of an organism due to internal physiological deterioration" (Rose 1991). As pointed out by Rose all definitions tend to color our thinking about a problem. For example, both definitions tend to stress the negative in aging. Aging can also include adaptation. Successful survival can be the result of an adaptation process to living as an organism learns (Poon 2011; Martin 2007).

The connection of the clock to aging is relevant to a number of theories of aging. Aging could be a timing phenomenon (Hagberg 2007), as with salmon or puberty, and hence the clock may have a rather direct relation to aging, being part of the programmed aging process (Hagberg 2007). This programmed aging can include apoptosis or programmed cell death (Cohen et al. 2004).

Alternatively, aging could be due to mutational accumulation (Hagberg 2007; Szilard 1959), as the organism accumulates damage ('wear and tear'). This 'wear and tear' mutational accumulation phenomenon can be dramatically enhanced by mutations in genes that produce conditions for accelerating aging, providing

insights into the aging process (Schumacher et al. 2008) as well as possibly greater longevity (Nebel et al. 2009). Human neurons, for example, are thought to be particularly vulnerable to DNA damage during aging (Lu et al. 2004). Some of these repair processes are under clock control. The surprise is that even the cellular processes responsible for keeping this 'wear and tear' at the cellular level under control can be under clock control in diverse organisms varying from *N. crassa* to humans (Pregueiro et al. 2006; Collis et al. 2007). The organism may time the delivery of these repair processes to protect the DNA.

Finally, Hagberg (Pittendrigh and Minis 1972) has argued that aging may be tied into a stress-response. For example, the maintenance of genome integrity as individuals age appears to be compromised with, for example, the p53 DNA damage response attenuated in aging mammals (Simon et al. 2009). Others have argued that aging is related to oxidative stress (Melov et al. 2000) and other stresses, such as heat-shock (Fabrizio et al. 2001) or the retrograde response to mitochondrial dysfunction (Jazwinski 2011).

In the end aging may be both a matter of timing, stress, and mutational accumulation. In each of these theories of aging different parts of metabolism are invoked to provide the underlying mechanism of aging (Bass and Takahashi 2010; López-Otín et al. 2016).

10.1 What Is the Biological Clock?

A phenotype is said to have a biological clock if this phenotype has the following properties: (1) a circadian rhythm; (2) entrainment to light or temperature or some other zeitgeber; (3) robustness in period to variation in temperature within a physiological range for the organism (Brunner and Káldi 2008; Lakin-Thomas et al. 2011). Property (3) is called temperature compensation—whether warm or cold, the biological clock ticks at the same rate. Such biological clocks have been established in a variety of model systems ranging from *Neurospora crassa* to *Arabidopsis thaliana*, *C. elegans*, *Drosophila melanogaster*, *Mus musculus*, and *Homo sapiens* (Dunlap 1999). Again the caveat about this definition is that it colors the kinds of questions asked about the clock. Most work on the clock occurs at the macroscopic scale involving 10^7 cells or more. The definition may need to be recast at the single cell level where the behavior of oscillators is quite different (Ko et al. 2010). For example, at the single cell level it may no longer be reasonable to insist for each oscillator to have a circadian rhythm with the same intrinsic period.

Clock mechanisms in animals, plants, and fungi have similar design. The clock mechanism is organized into positive regulatory elements and negative regulatory elements (Dunlap 1999). The negative elements provide the oscillators for the system, and the positive elements activate the negative elements and other outputs for the clock. These positive and negative elements form at least one negative feedback loop in the clock genetic network, to drive the oscillations (Yu et al. 2007) (Fig. 10.1a). This core clock mechanism is tied to a variety of proteins and small

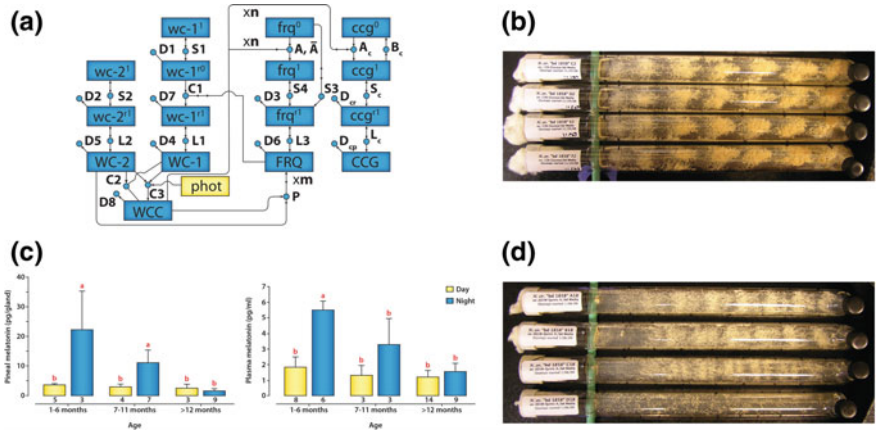

Fig. 10.1 The physiological readout (*i.e.* "clock phenotype") can be measured in a variety of ways depending on the organism with hormone levels and race tubes as examples. **a** The clock mechanism's genetic network in *N. crassa* consists of three genes, *frequency* (*frq*) (*i.e.*, the gene encoding the oscillator and negative feedback element), and *white-collar-1* (*wc-1*) and *white-collar-2* (*wc-2*) (i.e., the positive elements encoding two transcription factors together activating the oscillator gene *frq*). The mRNAs have an r as a superscript, and protein products are capitalized. The *circles* are reactions, and the *boxes* are reactants and products. Reactants have *arrows* going into a reaction; Products have *arrows* coming out of a reaction. A reactant/product with a *double arrow* is a catalyst. *Labels* denote the rate constants. Labels with an *S* denote a transcription reaction rate; Labels with an *L* denote a translation reaction rate. *Lollipops* denote decay reactions with rates labeled with a *D*. A *clock-controlled gene* (*ccg*) is an output of the clock mechanism regulated by the dimer WCC = WC-1/WC-2. From Dong et al. (2008). **b** Race tube readout of the clock after two serial transfers of the *band* (*bd*) mutant. **c** Melatonin readout in shrews of various ages day and night. From Magnanou et al. (2009). Levels of the ordinate are *below each bar*. Letters (**a** and **b**) label significantly different groups. **d** Race rube readout of the clock after 18 serial transfers of the *bd* mutant in (**b**)

molecules carrying information from the environment, such as light, temperature, and food availability. The working ensemble of models is currently supported by over 30,000 time points (Al-Omari et al. 2015) in *N. crassa*.

These outputs can involve many thousands of genes (Dong et al. 2008; Harmer et al. 2110; Wijnen et al. 2006). What is new and surprising is that some of these output genes or "*clock-controlled genes*" can actually feedback on the clock mechanism, providing, for example, inputs from metabolism on the functioning of the clock (Hurley et al. 2015).

In *N. crassa* the gene encoding the oscillator is *frequency frq* in Fig. 10.1a. This gene is transcribed at a rate S4 into an RNA frq^{r1}, which is then translated at a rate L3 into its protein product FRQ. When FRQ is high, it is dusk. All the labels in Fig. 10.1a do double duty—they are both labels for the reactions and the rate constants for the reactions. For example, reaction S1 has a rate constant S1 in

Table 10.1 Gene nomenclature for clock mechanism genes in different model systems

Species	Positive element	Negative element	Light-receptor
N. crassa	*wc-1*, *wc-2*	Frequency (*frq*)	*wc-1*[a]
D. melanogaster	Clock (clk), Cycle (cyc)	Period (per), timeless (tim)	Cryptochrome (cry)
M. musculus	BMAL1, CLOCK	PER, TIM	CRY

All genes listed are homologs and paralogs of each other
[a]The light receptor function in flies and mammals is broken out to a separate gene, not in *N. crassa*

Fig. 10.1a. When FRQ is low, it is dawn and yields the analog readout to the cell of time of day. The genes *wc-1* and *wc-2* are the positive elements in the clock that are transcribed (at rates S1 and S2) and translated (at rates L1 and L2) into their protein products WC-1 and WC-2. These two proteins dimerize to form the transcription factor WCC = WC-1/WC-2, which activates the oscillator gene frq^0 and many clock-controlled genes ccg^0. The gene *frq* acts as a negative element by producing a protein product FRQ deactivating WCC in the P reaction, closing the feedback loop. The oscillations in the clock are in part due to this negative feedback loop (Yu et al. 2007). The gene nomenclature for these positive and negative elements in summarized in Table 10.1 for several models systems.

10.2 Phenotypes that Respond to Both Aging and Circadian Rhythms

In order to facilitate the study of the connection between aging and the clock it is useful to have at least one phenotype that responds to both processes. Some examples of this are now given. This approach also requires that measuring the biological clock and aging is operationalized. From the definition of a biological clock a phenotype that is circadian is needed [property (1)]. A way to measure aging is also needed. Two aging measures have been widely used (Bitterman et al. 2003). One is *chronological lifespan*, i.e., how long an organism lives in a particular environ. The other measure is *replicative lifespan*, i.e., how long an organism can successfully replicate itself in a particular environment. The latter has been extensively used in *S. cerevisiae* (Jazwinski 2011). Ideally, the experimental setup would allow the measurement of both quantities along with circadian rhythms. Three examples are now given. The examples are chosen because they represent widely used ways to measure simultaneously aging and the clock in two kinds of systems, fungi and mammals. The third example illustrates a new approach to measuring the clock and aging at the single cell level, using the first two methodologies as controls.

10.3 Race Tubes

Circadian rhythms, chronological lifespan, and replicative lifespan can be measured together in the model fungal system, *N. crassa* (Case et al. 2014). Circadian rhythms are measured by means of race tubes (Davis 2000). Media is poured into glass tubes. The tubes are inoculated at one end with a mycelial plug or spores. Then the organism grows filamentously to the other end of the tube over several days. The bands observed (the organism producing spores) in the tube appear every ~22 h (Fig. 10.1b) and represent a biological clock. That is, they are circadian in behavior (Dharmananda 1980). These bands, the clock phenotype, entrain to light (Dong et al. 2008). They display temperature compensation (Liu et al. 1998). Most race tubes are run with a *band* (*bd*) mutation (Davis 2000). This band mutation was recently shown to be the homolog of a mammalian RAS proto-oncogene with the *bd* gene dually named *ras-1* in *N. crassa* (Belden et al. 2007a).

The same race tubes can also be used to measure chronological lifespan and replicative lifespan (Fig. 10.1b). The inoculum for each race tube can be split and used to start a culture under a particular condition (Case et al. 2016; Munkres and Furtek 1984). Then the live and dead cells can be counted over time to determine a survival curve and ultimately chronological lifespan. There are now fast and very accurate methods for counting cells that should replace traditional plating methods of counting cells (Berkes et al. 2012).

Once the culture reaches the end of the race tube, the culture can be serially transferred to another race tube (Fig. 10.1d). As this culture increases in replicative lifespan the regularity of banding diminishes. The number of serial transfers is a measure of replicative lifespan, or if more accuracy is needed, the total number of centimeters grown across serial transfers. Some cultures can only be transferred for a finite number times much like normal mammalian cells placed in tissue cultures (Bertrand et al. 1986; Bok et al. 2003). The replicative lifespan is thus analogous to a Hayflick limit in tissue culture.

10.4 Melatonin Levels in Shrews

A standard assay of a clock phenotype in mammals is melatonin levels associated with another clock phenotype, such as locomoter activity. An example of these clock phenotypes and how they can be used to study both clock and aging is found in shrews with their short generation time and high metabolic activity (Magnanou et al. 2009). The melatonin levels from blood samples and locomoter activity of shrews have a clear diurnal rhythm. Melatonin is a hormone that may be one major communication signal for the array of clocks that exist in mammalian tissue (Panda et al. 2002; McNamara et al. 2001).

Because of the short generation time, the melatonin levels can be monitored monthly with age (Fig. 10.1c). As the shrews age, the diurnal rhythms in melatonin

diminish. This is associated with diurnal locomoter activity. If shrews are given an explant to maintain melatonin levels, diurnal rhythms in behavior, such as activity, are maintained. Other kinds of clock outputs can also be observed to become more irregular with age in humans, such as, for example, arginine vasopressin (AVP) expression in suprachiasmatic nuclei, the seat of the master oscillator in humans (Hofman and Swaab 2006).

10.5 Aging Brains in Mice

A fundamental shift is taking place in the study of circadian rhythms in aging organisms, the shift from measurements on the clock phenotypes on the macroscopic scale to measurements on single cells (Ko et al. 2010). The master clock is thought to be housed in suprachiasmiatic nuclei (SCN) of the hypothalamus in mammals. The SCN is composed of about 10^4, 10^5 cells (Hofman and Swaab 2006; Yamaguchi et al. 2003). Understanding the clock and aging may ultimately depend on the behavior of individual cells in the SCN as they age (Farajnia et al. 2012).

In these kinds of studies measurements of locomoter activity, other behavioral measures and melatonin levels in mammals on the macroscopic scale would become the controls for single cells measurements of the clock. Farajni et al. (2012) measured the neuronal state of nuclei in the SCN by whole cell patch-clamp recordings on cells in young and aged controls of mice. Delayed-rectifier (DR) and A-type potassium currents were measured on single cells. The results were striking differences in the membrane potential of aged and unaged cells. The aged cells had experienced a loss of circadian modulation in function.

This kind of neuron measurement has been taken one step further to measure the relative levels of core clock gene products, such as PER1, in single cells of SCN in order to examine how the clock is functioning at the single cell level (Yamaguchi et al. 2003). The neuronal firing frequency and input resistance were linked to PER1 expression. Neurons possessing *per1* can be maintained in an excited state but do not fire (Belle et al. 2009). Single cell measurements have quantified the effects of *Per1* on neuronal activity through single cell measurements. In fact PER2 protein expression was the best predictor of whether or not an isolated cell from the SCN would behave in a circadian fashion (Webb et al. 2009). One would expect most cells to be highly synchronized in the SCN with respect to the clock as the master clock. Surprisingly, most cells in the SCN, when isolated, do not display a circadian rhythm.

One of the striking findings about the clock synchronization at the single cell level in the SCN is that age matters (Ono et al. 2013). Single cell and tissue level measurements were made on clock gene expression in neonates and adults. Adults lose synchronization at the tissue level but retain SCN cell synchronization at the cell level in the SCN. So as an organism ages, the organization of oscillators may change, leading to the proposal that the synchronization of oscillators is dynamic with age.

10.6 Metabolism Underlying the Link Between Aging and the Clock

The clock and aging are linked to thousands of genes in the genome (Zahn et al. 2007). Here we explore specific hypotheses about how aging and the clock are linked through metabolism (Bass and Takahashi 2010). Examining this linkage is facilitated by virtue of *N. crassa* being the only model circadian system for which the entire clock transcriptional network has been reconstructed without steady state assumptions (Al-Omari et al. 2015). All 2436 circadian genes have been placed in this clock genetic network with a new variable topology ensemble method (Al-Omari et al. 2015). The organization of the clock's entire transcriptional genetic network is shown in Fig. 10.2. The sequence of hypotheses below are not mutually exclusive and are placed within the context of the clock circadian network of *N. crassa*. The focus tends to be on fungal systems because most of what we know about metabolism in eukaryotes comes from the study of fungal systems, which enabled the original linkage of biochemistry and genetics through the work of Beadle and Tatum (Beadle and Tatum 1941).

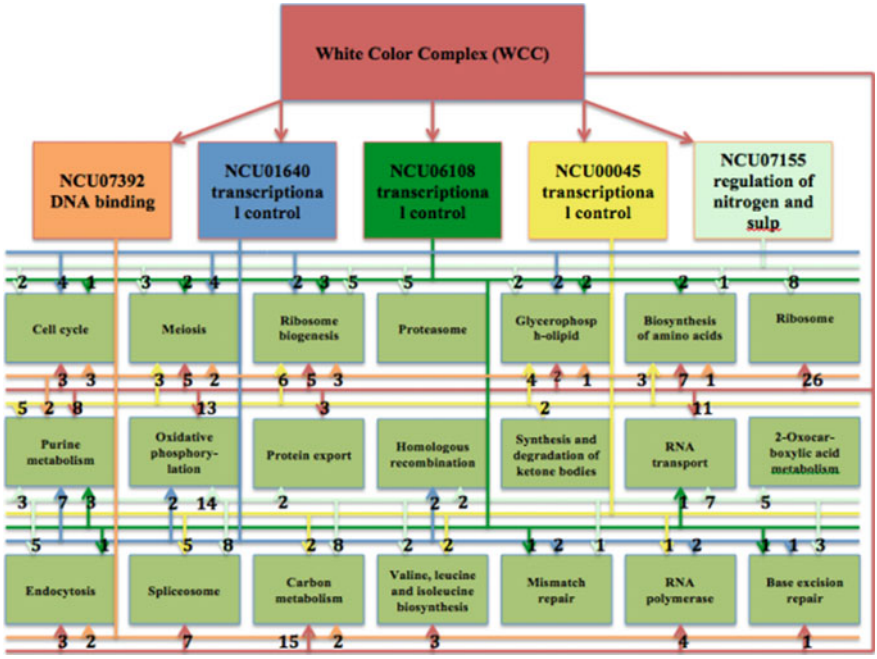

Fig. 10.2 Genetic network for the entire biological clock under the assumption of transcriptional control of *clock-controlled genes*. The figure is taken from Al-Omari et al. (2015). The *colored boxes* at the top are regulators. The *green boxes* are collections of inferred targets of the regulators. The regulator (NCU00045) is a repressor

10.7 Sphingolipid Metabolism May Link Aging and the Clock (Case et al. 2014)

In some aging theories, aging is viewed as a stress response (Hagberg 2007). In a similar vein the biological clock can also be viewed as a response to a periodic stress (Bennett et al. 2013). For example, *N. crassa* is very sensitive to UV light (Davis 2000). For other organisms, the activities that do not resonate with normal clock functioning may be the stress, such as being on swing shifts (Bass and Takahashi 2010). Whether aging or responding to the daily rhythm of the planet, it may be necessary for an organism to mobilize its metabolic reserves to respond to a stress. Lipids provide such a reserve, and some lipids also serve as key signaling molecules responding to stress (Hannun and Obeid 2011).

Metabolizing these lipid reserves may represent an adaptation linking the stress responses in aging and the clock. Some of these genes encoding products in lipid metabolism are highly conserved from yeast to mammals (Jazwinski et al. 2010; Jiang et al. 1998). The longevity assurance gene *LAG1* was the first longevity gene identified in *S. cerevisiae* (D'Mello et al. 1994). The *LAG1* gene and the *longevity gene cognate LAC1* both encode ceramide synthases (Guillas et al. 2001). In fact, in a double mutant *LAG1*, *LAC1* no sphingolipids appear to be produced (Schorling 2001). In microarray experiments on *N. crassa* it was found that the *N. crassa* homolog *lag-1* was shown to have a light entrainment response and to be under the transcriptional control of WCC (Dong et al. 2008) and was later shown to have a circadian rhythm in expression (Case et al. 2014). From the transcriptional network reconstruction of all six regulators the clock network is predicted to be controlling some aspect of lipid metabolism. The particular targets of these regulators are available (Al-Omari et al. 2015).

Earlier work in lipid metabolism has shown that not only did the clock mechanism control lipid metabolism, but there was a feedback mechanism of lipid metabolism on the clock (Lakin-Thomas and Brody 2000). For example, mutants in the lipid metabolism genes *choline require-1* (*chol-1*) and *chain elongation* (*cel*) had the dramatic effect of restoring circadian rhythms in the background of *white-collar-1 (wc-1)* or *frequency (frq)* mutants, two of the major clock genes in *N. crassa* that are homologs to *BMAL1* and *PERIOD1* (McClung et al. 1989; Crosthwaite et al. 1997). Later work, however, using a luciferase recorder driven by a *frq* promoter appeared to indicate that the metabolic oscillator associated with *chol-1* may act independently of the *frq*-based oscillator. However, the caveat is that a broader array of environmental conditions need to be explored in variable genetic backgrounds (Shi et al. 2007).

Motivated by this work and the findings from the microarray analysis (Dong et al. 2008), Case et al. (2014) showed that the mammalian RAS protooncogene homolog (*ras-1*) and *lag-1* as a double mutant could stop circadian rhythms as assayed by race tubes and that the double mutant *lag-1, ras-1* extended chronological lifespan and reduced replicative lifespan. A direct assay of clock function by RT-PCR also revealed that the cycling of the clock *frq* gene had a much longer

period in the *lag-1, ras-1* double mutant. The caveat to this work is that again background effects may be present, and luminescent or fluorescent recorders of clock genes were not used to give more than 2 days of temporal traces on clock mechanism genes (Castro-Longoria et al. 2010; Gooch et al. 2008).

Subsequent work on *lac-1* in *N. crassa* using a fluorescent recorder driven by a studied *clock-controlled gene-2* (*ccg-2*)'s promoter indicated that the double mutant *ras-1 (bd), lac-1* has a prolonged chronological lifespan and a feedback effect on the clock (Brunson et al. 2016). The double mutant *ras-1 (bd), lac-1* also shortened replicative lifespan. The limitation of this work was that the temporal traces of the fluorescent recorder were limited to two days, although the traces were done in triplicate.

The connection between the clock and longevity through sphingolipid metabolism is not simple. The effects of ceramide synthases on these processes depends on the level of expression of ceramide synthases (Jiang et al. 2004). Too little expression of *LAG1* was detrimental to viability in *S. cerevisiae*, and too much expression of *LAG1* was also detrimental. The effects of ceramides on aging are also nuanced. These findings were also borne out in *N. crassa* in studies of the levels of cell death as the concentration of phytoceramides was varied in the media, in which cells were stressed by the inhibition of glycolysis and heat-shock (Plesofsky et al. 2008). This result held up across a variety of mutant backgrounds including *bd*, *lac-1*^{KO} and *bd*, *lag-1*^{KO} strains (Brunson et al. 2016). This work provides evidence that the levels of ceramides are adaptive and subject to balancing natural selection. The conclusion is that one function of spingholipid metabolism is in regulating stress related cell death. It is likely that in *N. crassa* the effects on the clock are likely to be nuanced as well.

The nuanced effects of ceramides and their synthases also arise because of the many different types of ceramides being produced and many different long-chain base (LCB) substrates being used to make them. It is very difficult to separate the effects of one ceramide from another (Hannun and Obeid 2011). For example, in mouse neurons the "*LASS1* mutant" has been shown to cause neurodegeneration, but in the *LASS1* mutant it was not clear whether the levels of ceramides (C18) and derivatives or the levels of LCBs were the cause of neurodegeneration (Spassieva et al. 2016). By engineering another "LASS mutant" that is not normally expressed in neurons and is not able to make the C18 ceramide, they were able to eliminate neuronal cell death. Moreover, since the C18 ceramide was not altered by the transgene and the LCB levels were restored, the authors concluded that the loss of LCB was linked to neuronal cell death. They were able to replicate these findings with primary neurons in tissue culture treated with varying levels of LBCs. It would be interesting to know if LASS1 has similar effects in the SCN.

The data available on a variety of systems are then consistent with the hypothesis that the *RAS-1* and *LAG and LAC* genes and their homologs act epistatically and are involved in a transient stress response (Jazwinski 2011), which includes mechanisms for responding to periodically recurring stresses, through mechanisms such as apoptosis. Because ceramides are a diverse family of signaling molecules, genetic backgrounds may be important in this genetic interaction affecting the clock and aging.

10.8 SIRT1 Through Caloric Restriction Links Aging and the Clock

The *SIRT2* complex of genes are another highly conserved family of genes that affect longevity from *S. cerevisiae* (Kaeberlein et al. 1999) to mammalian systems (Satoh et al. 2013; Tissenbaum and Guarente 2001; Rogina and Helfand 2004). The *SIRT2* complex is so named after the *SIR2* gene in *S. cerevisiae*. Again, the robustness of the findings of SIRT2 effect on longevity need to be qualified by possible influence of genetic backgrounds (Burnett et al. 2011; Delaney et al. 2011). For example, the "*sirt2*" complex of four genes in *N. crassa* are all paralogous to human *SIRT1*. The SIRT2 gene may act upstream of the *RAS1*, *RAS2*, *LAG1*, and *LAC1* genes. The major effect of *SIRT1* (mammalian homolog) is thought to be on replicative lifespan. It has also been demonstrated to be linked to human longevity (Kim et al. 2012).

Early studies of aging in *N. crassa* implicated only the *age-1* cluster of genes in *N. crassa* (Munkres and Furtek 1984). These screens for aging genes were done under relaxed growth conditions. Other longevity genes were subsequently identified in *N. crassa* when the cells were stressed (Case et al. 2014). One hypothesis is that *SIRT1* is the major mediator of lifespan extension through caloric restriction (Howitz et al. 2003). One major phenotypic effect of *SIR2 (*mammalian SIRT1 homolog) in *S. cerevisiae* is to mediate life span extension through caloric restriction (Howitz et al. 2003). A number of compounds, such as resveratrol, were shown to promote expression of *SIR2* and hence replicative lifespan in *S. cerevisiae*. The mammalian *SIRT1* also mediated the switch from apoptosis to cell survival in a variety of mammalian tissues (Cohen et al. 2004). Its target in mammals was a DNA repair factor Ku70, which then sequesters the Bax factor away from mitochondria, resulting in cell survival. The gene *S. cerevisiae SIR2* is one of complex of genes that function as deacetylases (Kim et al. 1999) affecting diverse processes include rDNA recombination, genome stability, and silencing of silent mating type, rDNA, and subtelomeric regions. In *N. crassa* SIRT1 complex member *nst-1* has both effects on replicative lifespan and chronological lifespan (Fig. 10.3). Also the ATP-dependent chromatin remodeler *clockswitch* (*csw-1*) in *N. crassa* is necessary for clock function, but its exact biochemical function and affect on lifespan remain unknown (Belden et al. 2007b).

The SIRT1 protein also has a major impact on circadian rhythms in mammals (Asher et al. 2008). The mechanism appears to be through binding to the BMAL1/CLOCK complex, where it then deacetylates the PER2 protein, thereby affecting clock function. The longevity gene product SIRT1 is part of a major feedback loop that links metabolism with the clock mechanism (Nakahata et al. 2009; Ramsey et al. 2009). Nicotinamide adenine dinucleotide (NAD) acts as a metabolic signal produced by phosphoribosyltransferase (NAMPT). The levels of NAD are regulated in a circadian manner. When NAMPT is inhibited, then SIRT1 is released from the CLOCK:BMAL1 complex, promoting oscillations of the *PER2* gene. The feedback loop is closed by CLOCK binding to the *NAMPT* gene.

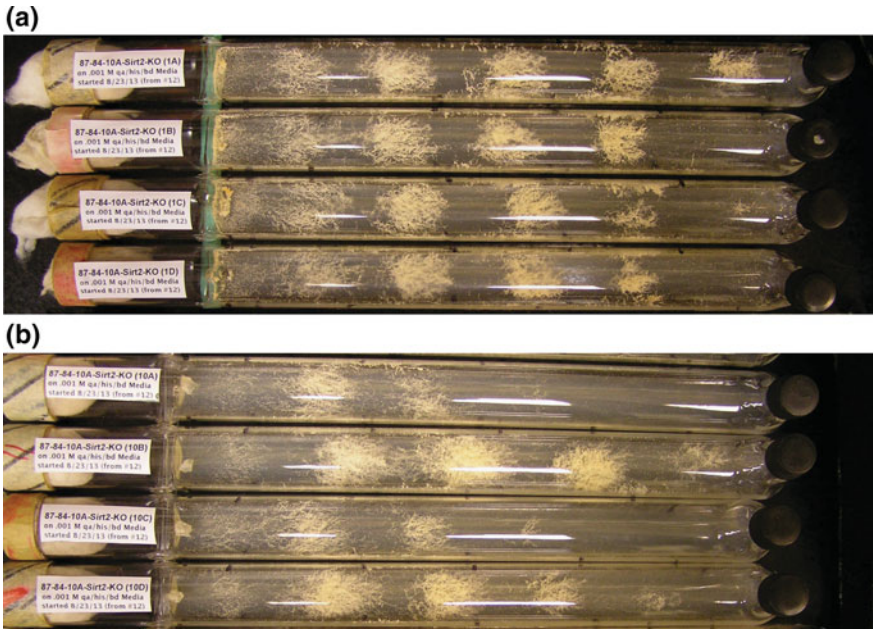

Fig. 10.3 The *sirt-1*, *bd*, *his-3* replicatively senesces after 10 serial transfers and the clock phenotype (physiological readout) becomes more irregular after 10 serial transfers as well. **a** Banding by 4 replicate race tubes at the start of the serial transfer experiment. **b** Banding by the same 4 replicate race tubes on the 10th serial transfer. The bands normally in a *bd* mutant appear every 22 h. Each of the four tubes are replicates of each other at the start of the serial transfer experiment

In replicatively aged cultures of *N. crassa* with a *sirt-1* knockout circadian rhythms were diminished as the cultures replicatively aged (Fig. 10.3). In *N. crassa* only 1 out of several of the *sir2*-related genes (NCU00523, the gene ID in *N. crassa*) was circadian and assigned to the regulation of mostly NCU6108 (Fig. 10.2) based on Fig. 9 in Al-Omari et al (2015) Nicotinate and nicotinamide metabolism has too few members to be included in Fig. 10.2.

In this way SIRT1 is able to signal programmed cell death as well as a circadian response to the stress response of caloric restriction. This environmental signal is an input to a closed feedback loop involving the clock and metabolism.

10.9 Oxidative Stress Links Aging and the Clock

Several authors have hypothesized that oxidative stress through Reactive Oxygen Species (ROS) links aging and the clock (Gyöngyösi and Káldi 2014; Yoshida et al. 2011). One of the oldest theories of aging involves ROS (Harman et al. 1955). One of the major model systems for understanding replicative senescence is the close

relative of *N. crassa*, *Podospora anserina* (Osiewacz 2002). This model of aging provides a wonderfully detailed story of how ROS contributes to replicative senescence through the retrograde response.

The story of replicative senescence has elements in common with the replicative senescence due to the insertion of a Kalilo element into mitochondrial DNA in *N. crassa* leading to senescence (Bertrand et al. 1986). The similarities are the insertion of a DNA element into mitochondrial DNA (mtDNA). The result is a loss of function of mtDNA. The loss of function is so rapid that growth of *P. anserina* in race tubes ceases prior to reaching the end of a race tube, and thus replicative lifespan is simply measured as the length of growth along the race tube. With the Kalilo element senescence usually takes 3–10 serial transfers (Bok et al. 2003).

The metabolic story in *P. anserina* begins with the identification of senescent DNAs (senDNAs) that target particular mtDNA genes (Wright et al. 1982). These senDNAs occur in a highly amplified form in senescent cultures. The next breakthrough came when the removal of subunit V of a functional COX5 gene product in respiratory complex IV (cytochrome C oxidase complex) was shown to lead to prolonged lifespan in *P. anserina* (Dufour et al. 2000). The removal of the COX5 gene product uncovered an alternative respiratory pathway involving an alternative oxidase (AOX). The presence of an alternative respiratory pathway was demonstrated, which produces lower ROS. Finally, the senDNAs were dramatically reduced in the removal of subunit V from COX5. With the reintroduction of a functional copy of the subunit, all of the expected phenotypes reappeared. This established a first formal connection between ROS and aging through the mitochondrial connection.

A subsequent discovery involved the *grisea* transcription factor (Borghouts et al. 1997). Three sequence mutations upstream of this gene led to extended lifespan, a reduction of senDNAs (now identified as pIDNAs) at the intron of the cytochrome C oxidase subunit I mtDNA gene (COI) and the concomitant mtDNA rearrangements induced. In subsequent work this gene was identified as a copper-modulated transcription factor that controlled the shift from oxidative phosphorylation depending on a copper requiring respiratory chain in complex IV to an alternate oxidative phosphorylation pathway only involving respiratory complex I-III and an alternate oxidase (AOX) requiring iron (Borghouts and Osiewacz 1998). This alternative AOX pathway led to increased lifespan by lowering ROS. There are many parallels between this retrograde response in *P. anserina* and those in *S. cerevisiae* (Jazwinski et al. 2011). In *N. crassa* the only homology to the *grisea* gene by BLASTX is to a hypothetical protein (NCU04773).

In contrast to the work on the mechanism linking ROS to aging in *P. anserina*, much of the work in *N. crassa* has focused on anti-oxidant enzymes, such as *catalase 1-catalase-4* (*cat-1-cat-4*), the four superoxide dismutases (*sod*), and the genes encoding the peroxiredoxins (NCU06031). These genes are all highly conserved (Rodriguez-Trelles et al. 2001; Edgar et al. 2012), with peroxiredoxins appearing as a phylogenetic marker for circadian rhythms in the tree of life. The *cat-1* gene has both a circadian rhythm and is light-responsive (Dong et al. 2008). The levels of peroxiredoxins is circadian in four kingdoms (Edgar et al. 2012).

The banding phenotype in *N. crassa* (Fig. 10.1) is also correlated with the diurnal cycle in ROS and may prove to be a clock phenotype (Yoshida et al. 2011). The enzyme activities of CAT and SOD correlated with the *age-1* mutant genotypes (Munkres et al. 1984).

The regulation of some of these antioxidant encoding genes are thought to be tied into the circadian system through a MAP-kinase cascade in *N. crassa* (Vitalini et al. 2007). There is evidence that two of the genes in this MAP-kinase cascade, osmotic mutants, *os-2* and *os-1*, are regulated by the clock mechanism (Dong et al. 2008; Vitalini et al. 2007). For example, the phosphorylated form of OS-2 is circadian, and the rhythm of the phosphorylated OS-2 form is lost in *frq*- or *wc-1*-mutant strains (Vitalini et al. 2007). Also *os-2* is reported to be under WCC control (Dong et al. 2008). The gene *os-1* is also circadian in expression (Dong et al. 2008). The tie of antioxidant expression and hence ROS levels to the circadian system appears to be partly through *os-2* (Yamashita et al. 2007; Noguchi et al. 2007), which has an effect on expression of CAT-1, CAT-3, and CAT-4. The connection of the clock mechanism is apparent in the reconstructed network through control of oxidative phosphorylation (Fig. 10.2). One transcription factor *ascopore lethal-1* (*als-1* or *atf1* or NCU01345) has been suggested to regulate the MAP kinase cascade (Yamashita et al. 2008) by analogy to *S. cerevisiae's* osmotic integrity kinase cascade (Gustin et al. 1998). This transcription factor also binds to the *clock-controlled gene-1* (*ccg-1*) (Lindgren 1994). This transcription factor does not appear in Fig. 10.2, but may be under the indirect regulation of one of the six transcription factors in Fig. 10.2 or be post-transcriptionally regulated by the clock mechanism.

The feedback of ROS on the clock mechanism is apparent as well. For example, ROS is circadian in level in the dark (*i.e.*, a D/D environment) (Yoshida et al. 2011). What is surprising is that the circadian rhythm is maintained even in the absence of *wc-1* or *frq* much as the oscillations in a *cel*- or *chol-1* mutant background (Lakin-Thomas and Brody 2000), suggesting the existence of another oscillator FRQ-less (FLO) in the system other than FRQ-based (FWO) oscillator (de Paula et al. 2006). The circadian oscillations in peroxiredoxins do not require the FWO oscillator as well (Edgar et al. 2012). The ROS levels were hypothesized to be a balance between the periodic destruction of ROS by catalases and peroxiredoxins and their regeneration by NADPH oxidase (Yoshida et al. 2011). They argue that these oscillations in ROS feedback on WCC levels. Alternatively, they could also constitute the basis of a FLO oscillator.

As we have already noted, there is evidence that *cat-1* is circadian (Dong et al. 2008; Yoshida et al. 2011). If another anti-oxidant encoding gene is introduced, *sod-1*, into wildtype, then banding is seen much like in the *band* phenotype in Fig. 10.1. The period is shorter by about 1 h. Moreover, light entrainment is much more pronounced in a *sod-1* background than in a *bd* background. Whether *sod-1* is affecting the FRQ-based clock mechanism or another oscillator is an open question.

One possibility is that *sod-1* and *cat-1* are part of a negative feedback to WCC in much the same way that as NAD is for BMAL1/CLOCK/SIRT1 (Nakahata et al. 2009; Ramsey et al. 2009). This feedback may be provided by hyperperoxides to

WCC from peroxiredoxins (Gyöngyösi and Káldi 2014). The gene encoding peroxiredoxins (NCU06031) is circadian (Dong et al. 2008). The primary regulator of NCU06031 is regulator NCU07155 (Al-Omari et al. 2015) (Fig. 10.2). This link closes the hypothesized feedback loop.

10.10 The Cell Cycle Links Aging and the Clock

In the population-based Georgia Centenarian Study cancer is the fourth highest cause of morbidity in centenarians after heart disease, dementia, and pneumonia (Arnold et al. 2010). The forkhead box O family (FOXO) and anaphase promoting complex (APC) genes play a highly conserved role in controlling the cell cycle and apoptosis from yeast to mammals (Postnikoff and Harkness 2012). They are also implicated in cancer. Initial examination of FOXO family members showed no effect on lifespan in yeast (Wei et al. 2008), but subsequent work found epistatic effects of *FKH1* and *FKH2* and *APC* on both chronological and replicative lifespan particularly when cells were stressed (Postnikoff et al. 2012). The effect of FOXO on aging has held up in other model systems (Hwangbo et al. 2004). The *FOXO3A* gene and its allelic variants has been linked to human longevity and a lower prevalence of cancer (Willcox et al. 2008). Both *FKH1* and *FKH2* are transcription factors in *S. cerevisiae* regulating the cell cycle (Zhu et al. 2000).

In *S. cerevisiae* by themselves *FKH1* and *FKH2* showed no effect on lifespan, but when a double mutant was created, then there were effects on both replicative and chronological lifespan (Postnikoff et al. 2012). This result is analogous to the epistatic effects of *ras-1* and *lag-1* on longevity in *N. crassa* (Case et al. 2014) and other systems (Jazwinski et al. 2010). This effect on lifespan was particularly dramatic when subject to caloric restriction (growth in H_2O) and ROS stress. The *FKH1* and *FKH2* act redundantly with each other to carry out this function. One of the predictions of this role of mediating stress through FOXO family members is that they should be more highly expressed under stress conditions. Both genes were overexpressed and were found to have both increased stress tolerance with an increase in chronological and replicative lifespan (Postnikoff et al. 2012).

The gene *APC* also has a highly conserved role in lifespan extension (Feser et al. 2010). One of the phenotypes of *APC* mutants are genomic instability, a hallmark of cancer. *APC* activity has been shown to be essential for promoting replicative and chronological lifespan in *S. cerevisiae* (Harkness et al. 2004). Under stress conditions some of the phenotypes in response to stress observed in *APC* mutants could be relieved by over-expression of either FKH1 or FKH2 (Postnikoff et al. 2012). The emerging model is that *APC* is a downstream target mediating the aging response of *FKH1* and *FKH2*. They work together as a redundant components to mediate the stress response as cells age.

In *N. crassa fkh1 (NCU00019)* is WCC responsive in the circadian network (Dong et al. 2008), but not circadian or light-responsive. The presence of cell cycle genes can be seen in Fig. 10.2, but *fkh1* is not part of this transcriptional network

(not being circadian). The gene *fkh1* does respond differentially to crossing versus natural media. The gene termed f*kh2* (NCU06173) does not show up as clock-associated (Dong et al. 2008). If the homolog to *APC1* is examined in *N. crassa (NCU05901)*, the gene is not clock-associated (Dong et al. 2008).

The cell cycle not only affects longevity, but the cell cycle is also strongly tied to circadian rhythms in several ways. As shown in Fig. 10.2, several cell cycle genes are part of the circadian network. One gene linking the clock to the cell cycle in *N. crassa* is the checkpoint kinase *prd-4* (Pregueiro et al. 2006). The *prd-4* gene was initially identified in a genetic screen because it shortened the period of the banding phenotype by 3 h (Gardner and Feldman 1981). It was later determined to be a checkpoint kinase in the cell cycle, a homolog of *CHK2 in H. sapiens* and *RAD53* in *S. cerevisiae*. The gene also conditionally feeds back on the circadian clock; if the organism encounters DNA damage, the hypothesis is that PRD-4 protein hyperphosphorylates the clock oscillator FRQ and resets the clock. This hypothesis was tested by treating cells with methylmethane sulfonate (MMS) to mimic ionizing radiation. The clock behaved as predicted. When FRQ is maximally phosphorylated at night, MMS has no effect; when FRQ is minimally phosphorylated at dawn, MMS has its maximum effect. A similar feedback loop exists in mammals (Gery et al. 2006). Recently these results have been incorporated into a genetic network linking the clock and cell cycle genes (Hong et al. 2014).

Another novel link between the cell cycle and circadian rhythms may be generalized and not involve any particular gene (Paijmans et al. 2016). The discrete replication events of the cell cycle may drive the circadian rhythms, and the circadian rhythms can sometimes become phase-locked to the cell cycle. Genes may intervene to prevent this from happening.

In summary the cell cycle becomes another pathway by which aging and the clock are linked. It displays several recurring themes. Genes interact epistatically to determine longevity. A feedback loop exists with cell cycle genes involved in longevity affecting the clock mechanism. Some of the genes in the pathway under consideration mediate stress responses, and their effects on the clock are conditional on the timing of stress responses.

10.11 Glucose Metabolism Links the Clock and Aging

Glucose levels in an organism are very likely to be subject to balancing selection. At one extreme caloric restriction is the only lifestyle intervention known to extend lifespan (López-Otín et al. 2016). At the other extreme too much glucose is associated with a cluster of phenotypes known as metabolic syndrome (Bass and Takahashi 2010; Perelis et al. 2015). Too much or too little glucose can affect the fitness of organisms from fission yeast to man (Roux et al. 2009). One glucose signaling pathway is, for example, intimately tied to the ability of an organism to become dormant (in *C. elegans*), and thereby extend lifespan through the process of dauer formation (Kenyon 2011).

One of the most ancient pathways to affect lifespan from worms to mammals is the IGF signaling pathway (Roux et al. 2009; Barbieri et al. 2003). The discovery of the importance of this pathway began in the *C. elegans* (Kenyon 2011). The pathway was later found to be linked through *daf-16* to the forkhead family discussed above under the role of the cell cycle. This pathway is conserved across animals, while eukaryotic microbes appear to utilize an early form (Barbieri et al. 2003). Barbieri et al. (2003) suggested a paradigm in which the glucose and IGF-1 signaling pathways have evolved and increased in complexity across the eukaryotes, with selection originally on the ability to postpone reproduction in times when resources are low. In *C. elegans*, mutations in *dauer formation-2* (*daf-2*) and *age-1* (which encodes a downstream PI3K in the insulin/IGF-1 pathway) are associated with longevity and slower aging (Barbieri et al. 2003).

The IGF signaling pathway is also one of the few pathways to have genes that consistently affect human longevity in several centenarian studies (Willcox et al. 2008; Suh et al. 2008; Bonafè et al. 2003). For example, in the Ashkenazi Jewish centenarians, their heterozygosity in the *IGF1* and *IGF1* receptor genes was higher in centenarians than a control group. Thus glucose through this signaling pathway has a highly conserved role in robustly determining lifespan.

A number of lifestyle and environmental factors have long been suspected to play a role both in longevity and the clock. For instance, a recent study showed that time-restricted feeding had numerous benefits, including behavioral, cardiac, and aging benefits in Drosophila (Gill et al. 2015). Moreover, a functional clock was necessary for cardiac benefits of diurnal feeding. The effects of the IGF signaling pathway has a deep interplay with the clock. The *BMAL1* and *CLOCK* genes, homologs of *wc-1* and *wc-2*, in β-cells in the pancreas orchestrate the production of diurnally varying levels of glucose and insulin in *M. musculus*, and this orchestration involves hundreds of genes in insulin production and secretion (exocytosis) (Perelis et al. 2015). For example, RNAs related to insulin secretion, Ca^{2+} signaling, cAMP/EPAC, and calmodulin-dependent protein kinases were circadian. The clock control in β-cells is local—BMAL1 and CLOCK proteins have their own enhancer sequences distal to the promoter of genes with circadian transcription. When *BMAL1 and CLOCK* are knocked out in β-cells, the mice experience diabetes. The functioning of the master clock in the SCN and glucose pumping rhythmically in mice are not enough to overcome the glucose intolerance generated by knocking out BMAL/CLOCK in β-cells.

The view of this mouse system has colored the view of glucose in circadian systems of fungi. There are at least six transcription factors that target carbon metabolism in *N. crassa* (Fig. 10.2) (Al-Omari et al. 2015). One of these is encoded by the gene *conidial separation 1* (*csp-1)*. It appears that CSP-1 protein can bind with a *wc-1* promoter and can repress *wc-1* in a glucose dependent manner (Sancar et al. 2012). When *csp-1* binds upstream of *wc-1* in WT in the presence of increased glucose, *wc-1* compensates by increased production of WC-1. Lower levels of glucose result in stronger transcriptional oscillations (Hurley et al. 2014), an interesting point in light of recent evidence that inhibition of the pentose phosphate pathway results in an increase in BMAL1/CLOCK binding sites and binding

affinity in mammals, particularly for clock-controlled genes (Rey et al. 2016). The question is why in an evolutionary sense does *N. crassa* have such a glucose-dependent compensation mechanism.

There are several feedback mechanisms evident in glucose metabolism. One example is knocking out *csp-1* in *N. crassa*. The result is a shortening of the period of circadian rhythms in *N. crassa* (Sancar et al. 2012). An equally spectacular example which is distinct from the NAD feedback backup mediated by SIRT1, is the levels of Nicotinamide Adenine dinucleotide phosphate (NADPH) produced by the Pentose Phosphate Pathway (Rey et al. 2016). NAPDH sets redox levels in the cell. NADPH levels are circadian. Inhibition of the Pentose Phosphate Pathway in flies, mice, and humans altered circadian rhythms both in phase and period. The effects have not been examined in fungi. In fact, the NADPH levels were shown to remodel the whole transcriptional architecture of mammalian cells through a distinct acetylase P300 acting on BMAL1/CLOCK.

Another role for glucose is in entrainment (property (2) of the clock). Entrainment is affected by pulses of different sugars when photosynthesis is disabled in *A. thaliana* (Haydon et al. 2013). It is very plausible that metabolic sugars would provide a signal to the clock mechanism in plants. In this system the morning expressed gene PSEUDO-RESPONSE REGULATOR 7 (*PRR7*) mutants have an effect like *csp-1* to alter the sensitivity to sugar, but in this case the PRR7 mutant affects entrainment.

We then have two very different roles for sugar hypothesized in the clock. One is a hypothesis of glucose compensation in which the clock is normally relatively insensitive to glucose over a range of glucose concentrations, and under the other hypothesis glucose acts as a zeitgeber or entrainment signal. It is very plausible that such a signal should exist in plants (or fungi!). What is striking about current analyses of glucose compensation models in fungi is that they are very brittle. That is, a narrow range of parameters in these models produce glucose compensation (Dovzhenok et al. 2015).

In summary glucose appears to have multiple effects on aging and the clock. There are at least two ways that glucose feeds back on the clock, one through NAD and another through NADP produced by the Pentose Phosphate pathway. There are two fundamentally different hypotheses about the role of glucose in circadian rhythms, namely in glucose compensation and glucose entrainment. Lastly, glucose can trigger chromatin remodeling of the transcriptional network by an aging clock through SIRT1 and P300 acetylases.

10.12 Summary and Recurring Themes on the Linkage of Clock and Aging

There are a variety of mechanisms by which the two timing phenomena of aging and circadian rhythms are linked. The linkages are likely to emerge from a detailed analysis of how metabolism is linked to aging and circadian rhythms (Hurley et al.

2015). This analysis is facilitated by the complete reconstruction of the transcriptional network for a circadian system in *N. crassa* (Al-Omari et al. 2015). The different linkages involve particular components of metabolism: sphingolipid metabolism, SIRT1 and caloric restriction, oxidative stress, cell cycle, and glucose metabolism. Most of these explanations for the linkage of the clock and aging involve epistatic interactions between genes, which implies the importance of examining genetic background effects. The signals produced that affect aging and circadian rhythms are diverse and include ceramides, glucose, NAD, and NADPH. Each of the hypothesized linkages of aging and the clock provided by components of metabolism involve feedback signals to the clock mechanism itself. In organisms that have multiple nuclei this raises the possibility that desynchronization of clocks in different nuclei and the concomitant inability to mount a coherent response to environmental signals may contribute directly to aging (Belancio et al. 2015).

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Chapter 11
Developing Circadian Therapeutics Against Age-Related Metabolic Decline

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Abstract Aging is characterized by a progressive decline in metabolism and physiology throughout the body, and the complex physiological basis is not fully understood. A key intrinsic mechanism to safeguard our physiological well-being is the circadian clock, the biological timer that coordinates diverse essential processes. Epidemiological and genetic studies in the past two decades have established a crucial role of the clock system in metabolic homeostasis and physiological health. Accumulating evidence also points to a functional link between clock decline (e.g., amplitude dampening) and metabolic aging. In this chapter, we review a close relationship among energy homeostasis, aging and the circadian clock. We also describe the current efforts to identify novel small-molecule therapeutics that enhance circadian and metabolic functions. Given that a weakened clock is in part responsible for the metabolic deterioration during aging, such circadian-based therapeutics could be exploited to decelerate metabolic decline and ultimately promote healthy aging.

Keywords Circadian clock · Age-related metabolic decline · Small molecules Circadian amplitude · Mitochondria

11.1 Introduction

Aging is characterized by gradual deterioration of metabolic, physiological and behavioral functions (Lopez-Otin et al. 2013). Despite increases in overall life expectancy, healthy life expectancy has grown more slowly, resulting in a prolonged duration of disability in many populations (Salomon et al. 2012). It was recently estimated that approximately 10 years of difference exist between overall

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and healthy life expectancies (Salomon et al. 2012), which means long-living populations may suffer debilitating health and lifestyle impediments, including sleep deficits and compromised energy and mobility, in their last decade of life. Therefore, extending healthspan and enhancing quality of life for the elderly is among the ultimate goals of research, medicine and healthcare.

Virtually all organisms have evolved a biological timer called the circadian clock that orchestrates essential metabolic and physiological processes, serving a fundamental role to maintain biological integrity and function (Takahashi et al. 2008; Liu et al. 2007; Belancio et al. 2014). An important discovery that emerged from epidemiological and laboratory studies in the past two decades is a crucial regulatory function of the clock in energy homeostasis and metabolic health (Rutter et al. 2002; Green et al. 2008; Asher and Schibler 2011; Gerhart-Hines and Lazar 2015). For example, circadian misalignment in human subjects, due to shiftwork schedule, environmental/lifestyle factors (e.g., light at night) or in laboratory settings, has been shown to closely associate with or cause dysregulation of energy balance including glucose and lipid homeostasis (Roenneberg et al. 2012; Scheer et al. 2009; Arendt 2010). Metabolic homeostasis is known to decline with aging, which in turn contributes to the impaired quality of life in the elderly (Lopez-Otin et al. 2016; Dominguez and Barbagallo 2016). Aging is also accompanied by circadian dysfunctions, particularly reduced amplitude or robustness in clock-regulated processes such as sleep/wake cycles and metabolic oscillation (Gibson et al. 2009; Brown et al. 2011; Banks et al. 2016). In this chapter, we first review a close relationship among metabolism, aging and circadian clock, which supports the notion that a weakened clock is likely responsible, at least in part, for the metabolic decline during aging. We also describe recently identified clock-targeting small molecules, particularly those showing promising protective efficacies against metabolic disorders (Hirota and Kay 2009; Nohara et al. 2015; He and Chen 2016). Such putative circadian therapeutics, especially the clock-enhancing molecules (Nohara et al. 2015; Chen et al. 2013), may be utilized to decelerate metabolic decline and promote healthy aging.

11.2 Metabolic Decline in Aging

Aging is known to associate with metabolic decline (Lopez-Otin et al. 2013; Dominguez and Barbagallo 2016). A set of key metabolic abnormalities, including central obesity, high blood glucose, hypertension, and abnormal triglyceride and cholesterol levels, are collectively referred to as the metabolic syndrome that predispose affected individuals toward life-threatening chronic diseases such as diabetes and cardiovascular disease. These metabolic risk factors all show increasing frequency and severity with age; for example, it is well documented that aging is closely associated with central obesity and altered body mass composition (Lopez-Otin et al. 2016; Dominguez and Barbagallo 2016; Sala et al. 2015). Furthermore, metabolic health is a core determinant of lifespan and healthspan.

Both genetic (e.g., inborn errors) and lifestyle factors (e.g., high-fat diet and sedentary) are known to disrupt metabolic homeostasis and accelerate aging (Vermeij et al. 2016; Dietz et al. 2015; Neufer et al. 2015). On the other hand, certain longevity loci (e.g. related to sphingomyelin) and the universally longevity-promoting caloric restriction (CR) strongly impact metabolism (Ma et al. 2015; Gonzalez-Covarrubias et al. 2013; Bishop and Guarente 2007). It has been proposed that attenuated nutrient sensing and mitochondrial functions are among the cardinal aging hallmarks (Lopez-Otin et al. 2013). Below we will highlight age-related attenuation in energy homeostasis and mitochondrial function.

11.2.1 Energy Homeostasis and Insulin Sensitivity

Total energy expenditure declines during aging, and the elderly population tend to display diminished energy expenditure (EE) and gross energy intake (EI) compared with young adults (Black et al. 1996). There is an age-related energy imbalance, with EI > EE in the elderly and EI < EE in young adults, resulting in higher body mass index (BMI) during aging (Pannemans and Westerterp 1995). Consistently, glucose and lipid homeostasis is also dysregulated with age. For example, early studies showed that the glucose disposal rate, which measures the duration after glucose intravenous injection until recovery to the fasting condition, was markedly lower in older subjects (95 min vs. 65 min in young subjects) (Silverstone et al. 1957; Davidson 1979). Aging is also accompanied by increased serum cholesterol levels (Rivnay et al. 1980), potentially due to reduction of cholesterol metabolism and bile acid synthesis (Bertolotti et al. 2007; Morgan et al. 2016; Labrie et al. 1997) and increase of hepatic cholesterol secretion (Einarsson et al. 1985).

Decline of insulin sensitivity in peripheral tissues significantly contributes to the impaired energy homeostasis during aging. In pioneering clamp studies, glucose metabolism (M), as measured by the glucose infusion rate (GIR), was found to diminish with age despite largely constant plasma insulin concentration (I) (Defronzo 1979). As a result, tissue sensitivity to insulin (M/I) was decreased in aged, and even mid-aged, subjects. Hepatic glucose production did not appear to change over age; rather, muscle is the main metabolic organ that maintains blood glucose levels and declines both structurally and functionally during aging (Defronzo 1979; Kenney and Munce 2003; Nair 2005). For example, attenuation of GIR in aged subjects indicates reduced insulin sensitivity in peripheral, primarily muscle, tissues (Rowe et al. 1983). In muscle, insulin mainly functions to regulate translocation of GLUT4 from cytosolic vesicles to cell surface to accelerate glucose uptake, thus activating glycolysis and OXPHOS activities to consume glucose (Kahn 1996). Since GLUT4 protein levels are constant in skeletal muscle under various metabolic conditions (Kahn 1996), insulin resistance in muscle primarily corresponds to the failure of GLUT4 translocation onto cell membrane.

Altered lipid homeostasis is strongly associated with insulin resistance during aging. Aged subjects showed exaggerated lipid deposition in muscle and liver,

which correlated with insulin resistance and glucose intolerance (Petersen et al. 2003). A major culprit for these metabolic abnormalities appears to be the reduction of mitochondrial TCA flux rate and ATP synthesis rate in the elderly (Petersen et al. 2003). As mentioned above, basal glucose production in liver was comparable between body composition-adjusted young and elderly subjects, whereas peripheral glucose metabolic rate in euglycemic-hyperinsulinemic clamp studies was significantly lower (∼40%) in elderly subjects (Petersen et al. 2003). These observations highlight the muscle as a primary metabolic organ to regulate age-dependent decline in metabolic rate related to diminishing mitochondrial function. Reduction in mitochondrial oxidative phosphorylation (OXPHOS), rather than lipolysis, was chiefly responsible for increased intramyocellular lipid contents, which in turn correlated with development of insulin resistance and type 2 diabetes (Petersen et al. 2004). Peroxisome proliferator-activated receptors (PPARs) gamma coactivator-α and -γ (PGC-1α and PGC-1γ), key regulators of fatty acid oxidation and mitochondrial function including OXPHOS in muscle, were reduced in elderly subjects (57–66 years) compared with young subjects (22–31 years) (Ling et al. 2004), suggesting a regulatory function integrating aging and energy homeostasis.

Accumulation of visceral fat mass is well documented in aging. Intra-abdominal fat (IAF) area was previously shown to exhibit positive correlation with age, whereas several other parameters (BMI, body weight, WHR, etc.) did not (Cefalu et al. 1995). Importantly, IAF area negatively correlated with insulin sensitivity (Cefalu et al. 1995), partly due to diminished secretion of the adipokine adiponectin. Adiponectin is mainly released from visceral adipocytes, and the amount released is negatively correlated with visceral adiposity. Adiponectin functions as an insulin sensitizer, acting on its receptors AdipoR1 and AdipoR2 to activate downstream signaling pathways including AMPK and PPARα (Kadowaki et al. 2006; Yamauchi et al. 2007; Ruderman et al. 2013). Therefore, reduction of adiponectin by the increasing visceral adiposity during aging exacerbates insulin resistance. In a recent screen for AdipoR agonists using C2C12 myotubes, a small molecule dubbed AdipoRon was found to bind to both AdipoR receptors and activate cognate signaling pathways, and physiological studies revealed improved glucose homeostasis and prolonged lifespan in mice fed with high-fat diet (HFD) (Okada-Iwabu et al. 2013). These observations support a role of visceral adiposity in metabolic aging, and highlight AdipoRs as an attractive anti-aging target.

Excessive lipid deposition in liver also leads to serious health consequences over age. For example, non-alcoholic fatty liver disease (NAFLD) is a progressive disease characterized by deterioration from hepatic lipid accumulation (steatosis) to non-alcoholic steatohepatitis (NASH), which can then further advance to cirrhosis and possibly hepatocellular carcinoma (HCC) (Sheedfar et al. 2013). In the population with NAFLD diagnosis, NASH and fibrosis development were observed at a much higher rate in the elderly group (>65 years old) than in the non-elderly group (<65 years old) (Noureddin et al. 2013). In addition to advanced NASH and fibrosis development, the elderly group showed cardiometabolic disorders including hypertension and cardiovascular disease which may directly contribute to their

mortalities (Noureddin et al. 2013). Overall, the mortality rate with liver disease drastically increases in humans over 45 years old (Schmucker 2005), and displays sexual dimorphism with higher prevalence in male than female in the same age range (Schmucker 2005; Regev 2001).

11.2.2 Mitochondrial Function and Dynamism

As described in the preceding paragraphs, mitochondrial dysfunction, particularly in skeletal muscle, plays a critical role in the decline of insulin sensitivity and energy homeostasis in the elderly (Petersen et al. 2003). Mitochondria is the primary site to produce adenosine triphosphate (ATP) for cellular energetic needs through oxidative phosphorylation (OXPHOS) (Wallace 2005; Bratic and Larsson 2013). However, OXPHOS also generates reactive oxygen species (ROS), with potential deleterious consequences including oxidative DNA damage, cytosolic stress response, altered protein structure (carbonyl derivatives, protein fragmentation, oxidation of amino acid side chain, etc.) and mitochondrial dysfunction (Wallace 2005; Bratic and Larsson 2013; Sastre et al. 2003; Balaban et al. 2005; Giorgio et al. 2007). In response to metabolic challenges including obesity, hyperlipidemia and tissue lipid accumulation, mitochondrial expression of uncoupling proteins (UCPs) and consequently non-ATP synthesis OXPHOS function are activated (Lowell and Shulman 2005), leading to oxidative stress and mitochondrial dysfunction and contributing to insulin resistance and type 2 diabetes (Lowell and Shulman 2005). Although low-level ROS stress can elicit beneficial compensatory responses (mitohormesis) (Lopez-Otin et al. 2013), cells tightly regulate mitochondrial quality and function and also control ROS production to minimize unwanted oxidative stress. In a cellular response to mitochondrial ROS production, the transcription factor NRF2 detaches from its inhibitory factor Keap1 and translocates into the nucleus, where it activates transcription of various genes encoding antioxidant molecules including thioredoxins (Txns) and glutathione peroxidases (Gpxs) to protect intracellular organelles especially mitochondria (Ma 2013). Likewise, mitochondrial catalase (MCAT) overexpression has also been found to inhibit ROS production, extend lifespan, and protect against H_2O_2-induced aconitase suppression (Schriner et al. 2005).

Mitochondrial quality control involves regulation of its dynamism, namely fission, fusion and mitophagy (Seo et al. 2010). Specifically, compromised mitochondria remove damaged components by fission, which are then digested by mitophagy to avoid release of cytochrome C from mitochondria and hence induction of cell death (Seo et al. 2010). New mitochondria are constantly synthesized to replenish mitochondrial mass and functional capacity. Such mitochondrial dynamism is well balanced in young, healthy cells. However, mitochondrial DNA (mtDNA) damage accumulates during the aging process, due to extended exposure to ROS or simply accumulation of mtDNA replication errors (Larsson 2010). In human skeletal muscle, it was also found that expression of *Mfn2* and

Drp1 genes, encoding key regulators of mitochondrial fusion and fission respectively, was reduced during aging (Crane et al. 2010). These and other age-related deterioration may lead to imbalance of mitochondrial dynamism, which provokes mitochondrial dysfunction, induces energy imbalance (Seo et al. 2010), and potentially leads to apoptotic cell death triggered by cytochrome C release from damaged mitochondria (Westermann 2010).

11.3 Circadian Regulation of Metabolism

11.3.1 Circadian Clock System and Metabolic Control

In the mammalian circadian clock system, a cell-autonomous molecular oscillator is present in essentially all cells in our body (Takahashi et al. 2008; Balsalobre et al. 1998). The oscillator consists of interlocked feedback loops, with both the primary core loop and the secondary stabilization loop containing positive and negative factors, including CLOCK/BMAL1 and PERIOD/CRYPTOCHROME (PER/CRY) for the former, and RORs and REV-ERBs for the latter. The two loops primarily intersect at *Bmal1* transcription, functioning as a temporal molecular apparatus to drive downstream clock-controlled gene oscillation throughout the day (Liu et al. 2007). At tissue and organismal levels, the cellular oscillators are coordinated via neuronal and hormonal signals by the central pacemaker, the suprachiasmatic nuclei (SCN) in the anterior hypothalamus, controlling gene expression and physiological functions in a tissue-specific manner (Welsh et al. 2010; Hogenesch and Ueda 2011).

Among the life processes closely controlled by the clock is metabolism (Rutter et al. 2002; Green et al. 2008; Asher and Schibler 2011). Diurnal metabolic cycles in photosynthetic organisms involves switching between photosynthesis and oxygen-averse nitrogen fixation. Likewise, alternating oxidative and reductive metabolism was also observed in an ultradian cycle in budding yeast, partly functioning to protect replicating DNAs from oxidative damage (Chen et al. 2007; Chen and McKnight 2007). In mammals, daily cycles can be divided into active/feeding and sleep/fasting phases (Bass and Takahashi 2010). Meals during the active phase lead to insulin secretion, functioning to promote anabolic metabolism. During sleep, other hormonal factors such as glucagon take over, stimulating energy store breakdown to maintain normal blood glucose levels. Circadian metabolic function was initially demonstrated by the radio-labelled deoxyglucose study showing drastically different glucose uptake rates depending on the time of the day (Schwartz and Gainer 1977). More recently, epidemiological and human-based laboratory studies have highlighted the importance of normal circadian/sleep cycles for metabolic homeostasis (Roenneberg et al. 2012; Arendt 2010). For example, even an short-term (10 days) enforced misalignment of circadian/sleep phases in a laboratory setting was sufficient to disturb various metabolic parameters such as blood glucose levels, consistent with the observed increased risk of metabolic syndrome in shift workers (Scheer et al. 2009).

Profiling studies have revealed principles governing the clock regulation of metabolism. A pioneering transcriptomic study demonstrated circadian oscillatory expression of key metabolic genes, encoding rate-limiting enzymes, nutrient sensors/transporters and transcription factors/cofactors that encompass all aspects of metabolism in both SCN and liver (Panda et al. 2002). Interestingly, the clock-controlled genes are highly tissue-specific, with only approximately 10% overlap between the two tissues. Consistent with these observations, several other studies also elucidated nodal, tissue-specific circadian control of metabolic pathways in metabolic tissues including muscle and adipose tissues (Yang et al. 2006; Storch et al. 2002; Miller et al. 2007; Eckel-Mahan et al. 2013). More recently, with improved temporal resolution, tissue coverage and algorithms, it was found that almost half of all genes display circadian expression in at least one tissue in the body (Zhang et al. 2014). Proteomic and metabolomic studies further underscore a prevalent role of the clock in metabolic regulation. In support of post-transcriptional regulation of the clock, significant numbers of liver proteins, although varying between 1/5 to almost half of total proteins measured in different studies, were found to exhibit cyclic accumulation patterns corresponding to non-oscillating mRNAs (Reddy et al. 2006; Robles et al. 2014). In a metabolomic study using human blood samples taken every 2 h over the circadian cycle, the constructed metabolite reference displayed excellent predictive capability, assigning circadian timing of test samples within approximately 3 h of precision (Minami et al. 2009). Consistent with a concerted metabolomic oscillation in circulation, additional studies have also revealed circadian metabolite fluctuation in various biological samples including tissues, organelles and breath (Asher and Sassone-Corsi 2015; Harfmann et al. 2016; Aviram et al. 2016; Martinez-Lozano Sinues et al. 2014). These profiling studies establish a complex yet coordinated network of metabolic flux as a function of circadian timing.

Research using circadian animal models has provided important insight into the function and mechanism of clock control of metabolism (Bass 2012). A classical mammalian clock mutant is the ClockΔ19 mutant mice, identified in a mouse *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis screen based on an observed long period (>28 h in homozygous mutant mice) followed by a loss of rhythm in extended exposure to constant darkness (Vitaterna et al. 1994). Physiological examination of ClockΔ19 mutant mice uncovered a multitude of metabolic disorders, including blunted feeding rhythms, hyperphagia, exaggerated obesity risk under HFD feeding or at older ages, elevated blood glucose levels and hypoinsulinaemia (Turek et al. 2005). In support of an important role of the circadian clock in metabolic regulation, almost all circadian mouse mutants characterized were found to suffer overlapping metabolic disorders and increased risks of metabolic and other related disease (Yu and Weaver 2011).

Bmal1 encodes the heterodimeric partner of CLOCK as the positive-acting transcription factor in the core loop. *Bmal1* is unique among the core clock genes in that single knockout of *Bmal1* is sufficient to disrupt the rhythm (Bunger et al. 2000) and cause severe pathophysiologies (Andrews et al. 2010; Shi et al. 2013), providing an ideal target for loss-of-function studies to delineate tissue-specific roles of circadian clocks (Table 11.1). For example, whereas mice lacking *Bmal1*

Table 11.1 Bmal1 knockout globally and in metabolic tissues

Knockout	Circadian phenotype	Lifespan	Metabolic phenotype	References
Global	Arrhythmic Non-circadian period range	Short	Age-dependent energy imbalance Reduced muscle fiber diameter Reduced mitochondrial mass and respiration	Bunger et al. (2000), Andrews et al. (2010), Shi et al. (2013), Lamia et al. (2008), Marcheva et al. (2010), Yang et al. (2016) Kondratov et al. (2006)
Pancreas-specific (Pdx-Cre)	Rhythmic Normal period	N.D.	Glucose intolerance, hyperglycemia and hyperlipidemia Small islet size	Marcheva et al. (2010), Sadacca et al. (2011)
Muscle-specific (Mlc1f–Cre)	Normal	Normal	Slightly increase of body weight at advanced age Muscle specific insulin insensitivity Increased muscle mass Normal fasting glucose, GTT and ITT Increased locomotor activity levels	Dyar et al. (2014)
Muscle-specific, inducible (HSA-Cre-ERT2)	Normal	N.D.	Muscular insulin resistance Hyperglycemia Glucose intolerance Serum triglyceride trended toward to decrease Male-specific lower fat mass	Harfmann et al. (2016), Dyar et al. (2014)
Liver-specific (Alb-cre)	N.D.	N.D.	Abnormal energy homeostasis Late onset of obesity (HFD) Reduce hepatic mitochondrial oxygen consumption rate (OCR)	Lamia et al. (2008), Jacobi et al. (2015)

(continued)

Table 11.1 (continued)

Knockout	Circadian phenotype	Lifespan	Metabolic phenotype	References
Adipocyte-specific (aP2-Cre, adiponectin-Cre)	Normal	N.D.	Obesity (RC, HFD) and adipocyte hypertrophy (RC) Hyperlipidemia hyperglycemia Normal Glucose infusion rate (GIR) and hepatic glucose production (HGP)	Paschos et al. (2012)
Heart-specific (aMHC-Cre)	Normal	Short	Heart hypertrophy	Kohsaka et al. (2014), Young et al. (2014), Ingle et al. (2015)

N.D. Not determined

developed glucose intolerance, body weight gain, adiposity and hypoinsulinemia at young age, liver-specific *Bmal1* knockout mice were found to display improved response to glucose challenge such as faster glucose clearance (Shi et al. 2013; Lamia et al. 2008). These observations, together with other related studies (Marcheva et al. 2010; Jacobi et al. 2015; Yang et al. 2016), suggest complex functional interactions between tissue clocks in an age-dependent manner. Similar to global *Bmal1* knockout, β-cell specific *Bmal1* knockout led to hyperglycemia and glucose intolerance with impaired glucose-stimulated insulin secretion (GSIS) (Marcheva et al. 2010; Sadacca et al. 2011). These results together indicate BMAL1 plays a critical role in β-cell function. In comparison, muscle-specific *Bmal1* knockout mice exhibited impaired insulin-dependent glucose uptake with reduced GLUT4 protein levels, attenuated glucose oxidation and shortened lifespan, although the exact phenotypes varied between different studies (Harfmann et al. 2016; Dyar et al. 2014). Cardiomyocyte-specific *Bmal1* knockout mice were previously shown to suffer dilated cardiomyopathy with hypotrophy of cardiomyocyte (Kohsaka et al. 2014), accelerated cardiac aging with diastolic dysfunction and age onset cardiomyopathy, which eventually led to early mortality (Young et al. 2014; Ingle et al. 2015). Finally, adipocyte-specific *Bmal1* knockout mice were found to be prone to diet-induced obesity and show hyperphagia and reduced energy expenditure (Paschos et al. 2012). Overall, mouse mutants harboring *Bmal1* knockout, both globally and in key metabolic tissues, constitute an invaluable genetic resource for understanding tissue-specific functions of *Bmal1* and circadian clocks (Table 11.1).

11.3.2 Reciprocal Regulation of the Clock by Metabolism

The clock and metabolism display a reciprocal regulation; in other words, while closely regulated by the clock, feeding or metabolism also resets the biological timing (Rutter et al. 2002). Initial restricted feeding (RF) experiments showed that food available only during daytime effectively altered the phase of peripheral clock gene expression over several days (Stokkan et al. 2001; Damiola et al. 2000), and the food anticipatory activity (FAA), presumably controlled by a food-entrainable oscillator (Stephan 2002; Mistlberger 2011), also emerged according to the new feeding schedule. Furthermore, in mice deficient in core clock components, FAA and gene expression in response to RF were modified compared with WT mice (Mistlberger 2011; Escobar et al. 2009). These observations clearly indicate a profound effect of meal and metabolism on the circadian clock. When coupled with HFD, daytime feeding led to greater body weight gain in mice compared with nighttime HFD feeding (Arble et al. 2009). In fact, time-restricted feeding (TRF) of mice during the nighttime has been shown to protect against the metabolic syndrome, in either preventive or therapeutic regimens, across multiple diets, and importantly also improved cardiac health and healthspan when applied to *Drosophila* (Hatori et al. 2012; Chaix et al. 2014; Gill et al. 2015). While this TRF paradigm impacts multiple pathways including leptin (Arble et al. 2011) and CREB, mTOR, and AMPK (Hatori et al. 2012), a fundamental theme is that excess nutrient intake during daytime, when mice are normally in an inactive/resting state, will lead to insufficient energy expenditure and instead promote energy storage as fat. In contrast, restricted nighttime feeding enhances the amplitude of circadian and metabolic rhythms, resulting in an improved energy balance.

Apart from timing, nutritional contents also influence our internal clocks (Kohsaka et al. 2007; Oosterman et al. 2015). In particular, HFD is known to alter the clock (Kohsaka et al. 2007; Pendergast et al. 2013). Under ad libitum HFD conditions, mice showed a slight increase in the free-running period length ($\sim$23.8 h) and altered phases in clock gene expression compared with regular chow-fed animals ($\sim$23.6 h) (Kohsaka et al. 2007). There was a significant reduction in the robustness or amplitude of circadian rhythms, including both clock gene oscillation in the periphery and feeding rhythms. This amplitude connection is intriguing as aging is known to dampen the clock amplitude (see below). The molecular and physiological mechanisms for HFD-induced circadian changes are not well understood, but likely involve multiple pathways. One possibility is that increased lipid contents may serve to modulate clock-related nuclear receptors. For example, PPAR nuclear receptors are known to have lipid ligands and function to drive *Rev-Erbs* through the peroxisome proliferator hormone response elements (PPRE), which in turn regulates *Bmal1* expression and circadian amplitude (Liu et al. 2007; Shulman and Mangelsdorf 2005; Canaple et al. 2006; Wang et al. 2008). Many other metabolites have also been shown to impinge on the circadian clock, including NAD (see below).

11.4 Age-Related Clock Decline and Its Impact on Metabolism

11.4.1 Circadian Decline and Amplitude Dampening in Aging

Although cardinal clock characteristics seemed to be maintained in aged subjects under entrainment conditions (Czeisler et al. 1999), dysregulation and misalignment was unmasked under free-running conditions (Weitzman et al. 1982). In humans, aging is associated with phase advance, most clearly evidenced by advanced sleep timing. Whereas one explanation for the earlier phase is period shortening (Weitzman et al. 1982), recent studies using human fibroblast cells showed a largely constant periodicity over age (Pagani et al. 2011). It was hypothesized that the phase advance in human sleep may instead result from a deficit in sleep homeostasis (Brown et al. 2011). In comparison, older mice (16.5 mo) showed delayed and more variable circadian activity onset under 12:12 Light:Dark (LD) cycles, and displayed longer circadian periods in constant darkness when compared with young mice (4 mo) (Valentinuzzi et al. 1997; Gutman et al. 2011; Nakamura et al. 2011; Nakamura et al. 2015). These results, together with studies in other species, suggest a species-dependent effect of aging on circadian period (Pagani et al. 2011; Valentinuzzi et al. 1997). However, in both humans and mice, old age correlates with prolonged transition to adapt to phase shift cues (Valentinuzzi et al. 1997; Sellix et al. 2012). It has been postulated that aging compromises circadian synchronization, weakening the intrinsic response to entrainment cues.

Consistent with an age-related deficit in synchronization, robustness, or amplitude, of rhythmic oscillations (the difference between peak and trough) is impaired (Gibson et al. 2009; Hofman and Swaab 2006). For example, rhythms in SCN firing rate, hormonal secretion (e.g., cortisol and melatonin) and body temperature are known to be compromised during aging (Hofman and Swaab 2006). Furthermore, sleep fragmentation, characterized by multiple short periods of sleep during the normal sleep phase and sleep during the usual active phase, is an established hallmark of aging and many age-related diseases including Alzheimer's disease, indicative of amplitude dampening of the sleep/wake cycle (Ju et al. 2014). At the molecular level, there is also broad dysregulation of clock gene expression (Banks et al. 2016; Kolker et al. 2003; Wyse and Coogan 2010). Interestingly, αMUPA transgenic mice, as a long-living mouse model, displayed 24-h circadian periodicity regardless of age (Gutman et al. 2011). These mice maintained robust behavioral and physiological rhythms, and core clock gene expression also showed an enhanced amplitude. In contrast, in *Bmal1*-null mouse mutant, which exhibit arrhythmic clock gene expression and defective clock-controlled physiological processes such as metabolism and activity (Bunger et al. 2000; Marcheva et al. 2010), premature aging phenotypes such as sarcopenia and cataract were observed (Kondratov et al. 2006; Kondratov et al. 2009). These studies suggest that

maintenance of circadian robustness, perhaps involving both clock gene oscillation as well as systemic synchronization (Ramkisoensing and Meijer 2015), may confer a life-extending effect.

11.4.2 Circadian Metabolic Decline During Aging

Given the circadian nature of metabolic processes, age-related metabolic decline should be evaluated, and often manifested, in a circadian- or sleep-related context. For example, glucose clearance clearly displays a circadian rhythm (Boden et al. 1999). Aged subjects were found to exhibit hyperglycemia and higher insulin secretion rates (ISR) compared with young subjects throughout the day (Frank et al. 1995). In accordance with age-related insulin resistance, body weight (BW) or body mass index (BMI) matched young subjects showed similar ISR with lower blood glucose levels compared with the older subjects (Frank et al. 1995).

One well-established circadian output marker is melatonin (Hofstra and de Weerd 2008), a sleep-regulating hormone whose synthesis pathway is governed by the clock (Benloucif et al. 2008). Aging attenuates circadian peak (and amplitude) as well as daily total secretion of melatonin (Sharma et al. 1989; Waldhauser et al. 1988; Pierpaoli and Regelson 1994). Diminished melatonin levels have been shown to correlate with lower sleep quality with decreased rapid eye movement (REM) and slow wave sleep and increased stage 2 non-REM sleep in the elderly (Weitzman et al. 1982; Munch et al. 2005). Sleep quality is important for metabolic homeostasis; in particular, slow-wave sleep significantly affects brain glucose utilization, sympathetic nervous activity and reciprocal growth hormone secretion (Nohara et al. 2015; Scheen et al. 1996). Conversely, impaired sleep quality or sleep deprivation reduces post-sleep increase in ISR, suggesting an adverse effect of poor sleep quality on energy homeostasis. Consistent with an important role of melatonin in metabolic homeostasis, rodent studies showed that melatonin administration improved obesity and diabetes phenotypes, and muscle insulin sensitivity, as measured by GIR, was also enhanced (Sartori et al. 2009; Agil et al. 2012). Since light exposure stimulates melatonin synthesis and production (Wurtman et al. 1963; Axelrod et al. 1964; Boivin and Czeisler 1998), which directly regulates the SCN clock in vitro (McArthur et al. 1991), appropriately timed bright light exposure may normalize circadian amplitude and phase and improve sleep quality and metabolism in older subjects (Czeisler et al. 1989; Czeisler et al. 1992; Arendt 2000).

Body temperature is a circadian output that shows a diurnal pattern with a dip during sleep (Hofstra and de Weerd 2008; Monk et al. 1995; Duffy et al. 2002). Heat production is a major component of energy homeostasis, and age-related deterioration in energy homeostasis negatively affects circadian body temperature rhythm. Indeed, the body temperature rhythm, especially circadian phase and amplitude, was markedly different in aged subjects compared with young- or middle-aged subjects, although the basal body temperature remained largely constant (Kenney and Munce 2003; Monk et al. 1995; Vitiello et al. 1986). Major

organs for heat production are liver and muscle, and age-associated decline in skeletal muscle mass and function is an important contributing factor for dampened energy homeostasis and thermogenesis (Kenney and Munce 2003).

11.4.3 Nutrient Sensing Pathways: At the Crossroad of Clock and Aging

CR is a universal means to extend lifespan in all species tested (Bishop and Guarente 2007), and known to deplete white adipose tissue, particularly the pro-inflammatory and diabetogenic visceral fat that accumulates over age (Finkel 2015). As in TRF and several other feeding/fasting paradigms, food consumption during CR is highly consolidated within a few hours, essentially elevating the amplitude of circadian food intake behavior (Longo and Panda 2016; Tevy et al. 2013); in accordance, CR enhances the amplitude of core clock gene expression (Katewa et al. 2016). CR involves several nutrient sensing pathways including AMPK, AKT and mTORC1, all of which have been reported to functionally interact with the clock (Bass 2012; Finkel 2015; Tevy et al. 2013; Jeong et al. 2015). For example, AMPK phosphorylates the circadian repressor CRY proteins to regulate their degradation and hence circadian periodicity (Lamia et al. 2009; Yoo et al. 2013). On the other hand, several functional features of AMPK, including subunit composition, subcellular localization, and substrate phosphorylation, have been shown to oscillate in a circadian manner (Jordan and Lamia 2013), suggesting a reciprocal control of AMPK by the circadian clock. Furthermore, previous studies in *Drosophila* showed that AKT and TOR regulate circadian periodicity via GSK3-β, a kinase with demonstrated roles in both *Drosophila* and mammalian circadian clocks (Zheng and Sehgal 2010). As in the case of AMPK, the circadian clock reciprocally regulates TOR protein turnover and pathway activities (Khapre et al. 2014; Zhang et al. 2014; Okazaki et al. 2014).

The Sirtuin (Sirt) family proteins are crucial regulators integrating energy homeostasis, clock and aging (Tevy et al. 2013; Imai and Guarente 2014). Silent information regulator 2 (Sir2) in the yeast was initially discovered as a NAD-dependent deacetylase whose overexpression and deletion were found to extend and shorten yeast lifespan respectively (Kaeberlein et al. 1999). Seven Sir2 homologues (Sirt1-7) were subsequently identified in mammals (Chang and Guarente 2014), and various Sirt1 activating small molecules (e.g., Resveratrol, Quercetin or Butein) were reported to extend lifespan (Hubbard and Sinclair 2014). Several mammalian Sirtuins have been implicated in circadian regulation of metabolism (Asher et al. 2008; Nakahata et al. 2008; Masri et al. 2014; Peek et al. 2013). For example, SIRT1 directly deacetylates core clock components including BMAL1 and PER2, regulating their molecular function and expression of clock-controlled genes (Asher et al. 2008; Nakahata et al. 2008). In a more recent study examining a brain-specific function of SIRT1, SIRT1 was found to interact

with PGC-1α to control *Clock* and *Bmal1* gene expression in the SCN, subsequently regulating CLOCK/BMAL1 target genes (Chang and Guarente 2013). Furthermore, brain-specific Sirt1 knockout (BSKO) mice mimicked aging-like circadian phenotypes including lower activity levels, delayed response to circadian entrainment and dampened circadian clock genes amplitude and expression levels, whereas brain-specific Sirt1 overexpression (BSTG) mice showed protective effects against these aging phenotypes (Chang and Guarente 2013). Expression of the gene encoding nicotinamide phosphoribosyltransferase (NAMPT), a rate-limiting enzyme for NAD synthesis, is also controlled by CLOCK/BMAL1 and shows an oscillatory pattern over the circadian cycle (Ramsey et al. 2009; Nakahata et al. 2009). NAMPT synthesizes nicotinamide mononucleotide (NMN), a NAD precursor, in mammals and plays a key role of mitochondrial function and cellular metabolism (Garten et al. 2015). It has been shown that cellular levels of NAD exhibit an age-associated reduction (Ramsey et al. 2008). In a recent study where old mice were fed with the NAD precursor nicotinamide riboside (NR), Sirtuin activities, energy metabolism and stem cell/tissue maintenance were all improved as a result (Zhang et al. 2016). These studies strongly support a central role of the NAD-Sirtuin axis as a crucial regulatory pathway and drug target for energy metabolism, circadian rhythms and aging.

11.5 Manipulating the Circadian Clock by Small Molecules to Retard Metabolic Aging

11.5.1 Chronotherapy Versus Clock-Modulating Compounds as Novel Chronotherapeutics

Given the important role of the clock in metabolic and physiological well-being, there is an increasing interest to exploit the clock machinery for health benefits. Classical chronotherapy (Levi and Schibler 2007) focuses on identifying the optimal temporal window for therapeutic administration to maximize the therapeutic index, namely efficacy vs. toxicity. More than half of top drugs, many of which target chronic diseases that affect lifespan, act on protein targets encoded by cyclically expressed genes (Zhang et al. 2014). Coupled with circadian regulation of drug metabolism (Levi and Schibler 2007), this finding indicates that the pharmacokinetic and pharmacodynamics profiles of blockbuster drugs likely vary according to circadian time of administration. Many disease episodes or symptoms also show distinct circadian preference such as early-morning heart attacks and nocturnal asthma. In accordance, chronotherapies involving timed therapeutic administration has been reported for various diseases including cancer (Levi and Schibler 2007; Antoch and Kondratov 2013). In a mechanistic study (Gorbacheva et al. 2005), the sensitivity to the chemotherapeutic drug cyclophosphamide has been linked to the functional state of the central circadian transcription factor

CLOCK/BMAL1. Genetic mouse mutants with varying strength of CLOCK/BMAL1 (the positive arm of the core loop) were treated with the drug, and cell toxicity was found to well correlate with the expression and/or activity of CLOCK/BMAL1.

A distinct strategy to capitalize on the regulatory role of the clock in disease and aging is to manipulate the clock or clock components (Nohara et al. 2015; Chen et al. 2013; Wallach and Kramer 2015; Schroeder and Colwell 2013) (Fig. 11.1). In other words, rather than aligning the timing of therapy with the intrinsic rhythm, one can alter or enhance the intrinsic rhythm to achieve desired outcome. A number of behavioral or dietary manipulations are known to alter the clock, such as light exposure (Belancio et al. 2014; Banks et al. 2016; Fonken and Nelson 2014), exercise (Schroder and Esser 2013) as well as feeding/fasting regimens (Longo and Panda 2016). In particular, time-restricted feeding (TRF) was recently shown to improve sleep and body weight control and delay cardiac aging and healthy aging in *Drosophila* (Gill et al. 2015). Expression analysis identified TRF-dependent activation of genes involved in circadian rhythms and the mitochondrial electron

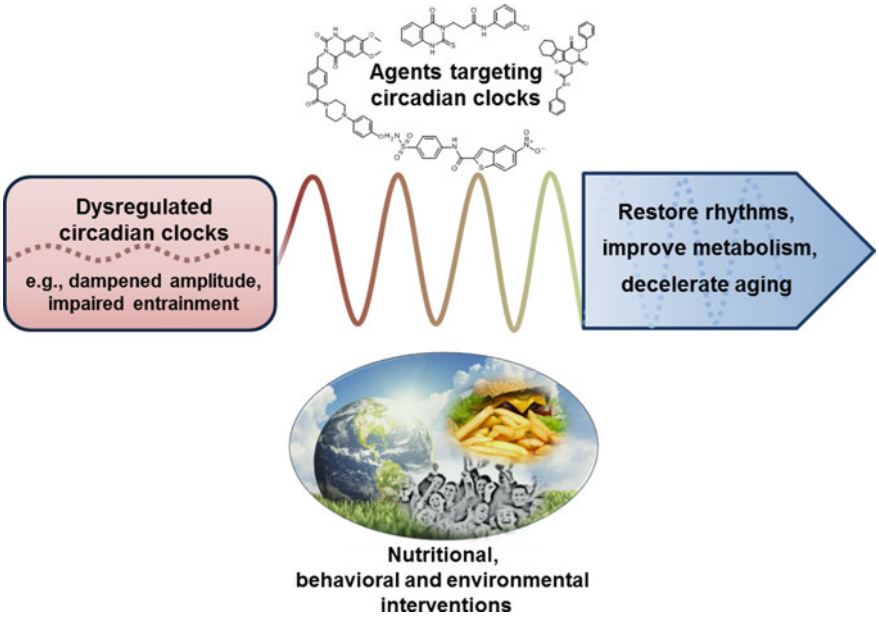

Fig. 11.1 Circadian interventions to delay metabolic aging. The circadian clock plays a key role in metabolic regulation, and dysregulation of the clock, particularly dampening of circadian amplitude, is closely associated with age-related metabolic decline. In addition to directly manipulating particular metabolic regulators, various interventional strategies, including nutritional (e.g., CR), behavioral (e.g., exercise), environmental (e.g., lighting), and pharmacological agents (e.g., clock-enhancing small molecules, or CEMs), have shown efficacies to enhance circadian rhythms, promote metabolic homeostasis, and ultimately delay metabolic aging. Adapted from Nohara et al. (2015)

transport chain complexes. Similar to TRF, CR temporally consolidated food intake, potently enhanced the expression and amplitude of core clock genes and consequently altered lipid homeostasis, all contributing to its life-extension effects (Longo and Panda 2016; Katewa et al. 2016). Finally, studies conducted in group care facilities showed a beneficial effect of bright light and melatonin, both major circadian synchronizers that strengthen rhythms, on cognition and mood in the elderly (Riemersma-van der Lek et al. 2008). Moreover, lifespan extension was observed following melatonin administration to mice, even for melatonin-deficient C57BL/6 mice, as well as pineal graft implantation from young to old mice (Pierpaoli and Regelson 1994). These studies collectively provide strong evidence that enhancing the molecular and physiological rhythms via dietary and behavioral means can improve metabolism/physiology and promote healthy aging.

To circumvent common compliance issues inherent in behavioral/dietary/lifestyle interventions, an attractive strategy involves chemical compounds capable of clock manipulation for convenient and consistent intake over a prolong period of time (Fig. 11.1). Two major modes of discovering circadian modifier compounds have been reported, namely unbiased phenotypic screens of large compound libraries and targeted approaches focusing on particular clock components (He and Chen 2016; Chen et al. 2013). Small molecule identification via phenotypic screens entails high-throughput-based circadian reporter assays, most commonly driven by *Per2* or *Bmal1* promoters (Hirota and Kay 2009; Chen et al. 2012; Jones et al. 2013; Isojima et al. 2009). However, protein targets have not been experimentally elucidated for a number of compounds (He and Chen 2016), which remains a major challenge toward translational applications. Significant progress, however, has been made in targeting several clock components described below.

11.5.2 Circadian and Physiological Efficacies of Clock-Modulating Small Molecules

The remarkable precision in circadian periodicity measurement facilitated the discovery of a large number of compounds with strong effects on circadian period length (Chen et al. 2013). Given the predominant regulatory role of casein kinase I (CKI) in circadian periodicity, various small molecules showing pronounced period-lengthening effects were identified as CKI inhibitors (Chen et al. 2012; Isojima et al. 2009; Hirota et al. 2010). These studies also affirmed the notion that PER protein half-life is a key determinant of circadian period length (Gallego and Virshup 2007). Existing CKI inhibitors typically show cross-inhibition of multiple CKI isoforms (e.g. CKIδ and CKIε) (Isojima et al. 2009). In contrast, the compound PF-4800567 was developed as an isoform-specific inhibitor of CKIε, yet interestingly displayed minimal effects on circadian periodicity (Walton et al. 2009), revealing a major role of CKIδ in clock regulation. More recent studies have further exploit the CKIε-specific ligand in clock-related pathophysiology such as substance abuse (Bryant et al. 2012).

CRY proteins, dimerizing with PERs in the negative arm of the core loop, are also subject to elaborate protein stability control, and mutations in two paralogous E3 ligases for CRYs have been discovered in mouse forward genetic screens to cause period changes (Yoo et al. 2013). Unbiased phenotypic screening revealed a potent period-lengthening carbazole compound, KL001 (Hirota et al. 2012). Biochemical studies revealed CRYs as the protein target of KL001, and KL001 binding interfered with CRY ubiquitination and subsequent degradation. Recently, pilot experiments showed that derivatives of KL001 improved mouse glucose tolerance (Humphries et al. 2016), suggesting a potential metabolic effect of activating CRYs.

REV-ERB and ROR receptor families, in the secondary loop of the circadian oscillator, function in an antagonistic manner to regulate RORE-mediated transcription of target genes such as *Bmal1* (Liu et al. 2007). These multi-functional receptors play a predominant role in circadian amplitude regulation, and recent studies have demonstrated novel amplitude regulatory mechanisms involving chromatin loading and posttranslational protein turnover (Zhu et al. 2015; Zhao et al. 2016). A number of ligands for REV-ERBs and RORs have been reported in recent years (He and Chen 2016; Kojetin and Burris 2014; He et al. 2016). Several studies focused on SR9011, a REV-ERB agonist developed through targeted chemical modification (Solt et al. 2012). SR9011 modified clock gene expression in metabolically active tissues and acutely inhibited circadian wheel-running behavior. Diet-induced obese mice fed with SR9011 showed improved metabolism including body weight and glucose/lipid contents in circulation (Solt et al. 2012). SR9011 has also been found to increase wakefulness in treated mice and reciprocally suppress REM and slow-wave sleep (Banerjee et al. 2014). An anxiolytic effect of SR9011 was demonstrated in WT but not *Rev-erbβ* knockout mice. These results suggest a therapeutic potential of REV-ERB agonists in modulating circadian/sleep-related metabolism and behavior.

Diverse small molecules targeting RORs have also been described (He and Chen 2016; He et al. 2016; Helleboid et al. 2014; Kallen et al. 2002; Wang et al. 2010; Jin et al. 2010) (Table 11.2). Besides its circadian activator function in the secondary loop, genetic and genomic studies have revealed important roles of RORs in development, neuronal system, motor function, metabolism and immunity (Jetten et al. 2013; Chang et al. 2012). Recent advances elucidated cholesterol intermediate metabolites as possible endogenous ligands (Santori et al. 2015), although the physiological function of the interaction, especially in the circadian system, is currently unknown. Due to a predominant role of RORγt in autoimmune disease, a large number of studies focused on its inhibitors and their application in immune response (Chang et al. 2012; Xu et al. 2011; Huh et al. 2011). How these RORγt ligands affect circadian and metabolic functions requires further investigation. In a recent study, a chemically derived antagonist of RORγ was found to display corrective effects against obesity and insulin resistance, likely involving stimulation of fatty acid oxidation and thermogenic activity (Chang et al. 2015).

Table 11.2 Natural ligands for RORα and RORγ

Natural ligands	Target isoforms	Activity	References
Nobiletin	RORα, RORγ	Agonist	Zhao et al. (2016)
Cholesterol Cholesterol sulphate	RORα, RORγ	Agonist	Banerjee et al. (2014), Kallen et al. (2002)
7-hydroxycholesterols	RORα, RORγ	Inverse agonist	Helleboid et al. (2014)
20α-hydroxycholesterol 22R-hydroxycholesterol 25-hydroxycholesterol	RORγ	Agonist	Kallen et al. (2002)
Cholesterol biosynthetic intermediates (CBIs)	RORγ	Agonist	Jetten et al. (2013)
Ursolic acid	RORγ	Inverse agonist	Chang et al. (2012)
Digoxin	RORγ	Inverse agonist	Santori et al. (2015)
Neoruscogenin (25S)-ruscogenin	RORα	Agonist	Solt et al. (2012)

11.5.3 Clock-Enhancing Molecules (CEMs) and Nobiletin (NOB)

The clock is intrinsically a self-limiting machine, with a myriad of check-and-balance mechanisms governing its periodicity. Previously it has been shown that the stoichiometric ratio between the stimulatory and repressive components in the core oscillator is important for determining the overall robustness of the clock (Lee et al. 2011). This, however, leads to the conundrum that activating or inhibiting a particular clock component beyond a certain threshold will invariably encounter compensatory responses in the oscillator that diminish or even abrogate the intended effects. Rather than focusing on a particular clock molecule, we hypothesize that amplitude readout may constitute a desirable endpoint to identify chemical modifiers that enhance the overall oscillation robustness. Employing a cell-based phenotypic screening approach, we have reported a group of clock amplitude-enhancing small molecules dubbed CEMs (Chen et al. 2012, 2013). In the first screen of 200,000 compounds, 4 CEMs were found to enhance cellular and tissue reporter rhythms in both WT and ClockΔ19/+ heterozygous mutant backgrounds (Chen et al. 2012). In contrast, these CEMs (CEM1-4) failed to rejuvenate rhythms in ClockΔ19/Δ19 homozygous or *Bmal1*-null cells where the oscillators are essentially broken (Chen et al. 2013). Of note, however, it is exceedingly rare for humans to have completely disrupted clocks. CEM3 exhibits a unique quality of enhancing the reporter rhythms of the central pacemaker SCN, known to be highly resistant to genetic and environmental perturbations as a result of robust neuronal

coupling (Liu et al. 2007). Given the dampened circadian amplitude associated with chronic diseases and aging, the clock-enhancing efficacy of CEMs may protect against related physiological decline and improve well-being in animals and humans.

The validity of this strategy is exemplified in recent studies of Nobiletin (NOB), a naturally occurring flavonoid functioning as a novel CEM (He et al. 2016; Nohara et al. 2015) (Fig. 11.2). NOB was identified through a cell-based screen showing robust enhancing activities in circadian reporter cells, with an EC50 in the low μM range. NOB is a major constituent flavonoid found in citrus peels, which have been used in cuisine and traditional medicine throughout the world for millennia. NOB is polymethoxylated, exhibiting a favorable pharmacokinetic profile devoid of significant toxicity (Evans et al. 2012). Previous studies have reported diverse biological activities, including anti-oxidant, anti-inflammatory, and anti-tumor efficacies (Lee et al. 2013; Matsuzaki et al. 2008; Mulvihill et al. 2011; Walle 2007), yet those effects were often shown at high μM concentrations and its molecular mechanism of action and direct protein targets were unknown. In our study using two mouse metabolic disease models, namely the HFD-induced obese and *db/db* diabetic mice, NOB was found to very effectively blunt body weight gain without altering food intake, enhance energy expenditure and circadian activity, improve glucose and insulin tolerance, and reduce lipid content in circulation and in liver (He et al. 2016). Furthermore, NOB also lowered serum ammonia levels under varying diet conditions, and appeared to enhance urea cycle gene expression and function under HFD feeding conditions (Nohara et al. 2015). These metabolic effects are clock-dependent, as ClockΔ19 homozygous mutant mice showed no or much diminished response to NOB. Transcriptomic and molecular analyses revealed prevalent remodeling of the liver metabolic network, affecting key metabolic pathways (e.g., lipid metabolism and mitochondrial respiration) and effectors such as PPARγ and C/EBP.

Filter binding assays as well as functional studies including mammalian one-hybrid assays identified RORs as direct targets of NOB (He et al. 2016). NOB ostensibly functions as an ROR agonist, activating *Bmal1* and other ROR target gene expression. The activation of core clock genes and downstream output genes are generally moderate, consistent with the limit-cycle nature of the clock. Apart from competing with REV-ERBs to activate target gene expression, a recent study revealed a novel mechanism where RORs function to facilitate recruitment of REV-ERBs during the repressive phase, thereby enhancing the overall circadian amplitude (Zhu et al. 2015). Although the exact clock-enhancing mechanism of NOB and the output pathway leading to enhancement of energy homeostasis require further work, the identification of NOB as a CEM with in vivo efficacy in metabolic disease models validates the strategy of exploiting small-molecule therapeutics to favorably manipulate circadian timing (Bass 2016).

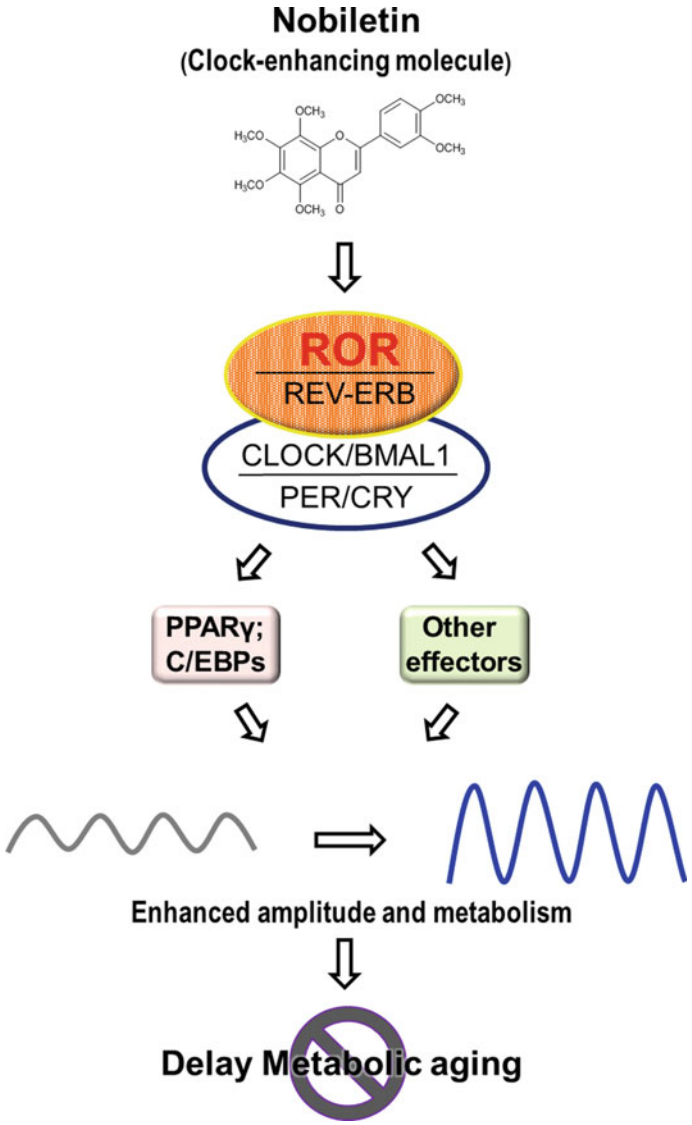

Fig. 11.2 A naturally-occurring polymethoxylated flavonoid, Nobiletin (NOB), was recently found to enhance circadian rhythms by targeting the ROR receptors in the secondary loop of the circadian oscillator. Through cellular effectors including PPARγ and C/EBPs, NOB broadly remodels metabolic networks in mice, potentially serving to decelerate age-related metabolic decline

11.6 Experimental Investigation of Clock-Modulating Molecules Against Aging

Distinct animal models can be employed to test the efficacy of clock modulators against metabolic aging, including naturally aged mice or those under stress conditions such as metabolic challenge and/or with genetic predisposing mutations toward age-related diseases. For example, it has been shown that HFD feeding can lead to shortened lifespan and healthspan (Okada-Iwabu et al. 2013). Key aspects of experimental design include timing, dose and route of administration. Given the expected temporal efficacy of circadian therapeutics, the timing of administration should be chosen based on both their pharmacokinetic features and the circadian expression and/or activity of protein targets. The typical requirement for chronic, or even life-long, treatment in aging studies also imposes a considerable constraint on treatment route and maximum tolerated dose. A straightforward strategy is to incorporate the compound of interest in food or water. Other than lifespan, various assays can be conducted to measure molecular, behavioral and metabolic readouts, in a circadian context whenever possible. Deterioration or fragmentation of behavioral rhythms over age is well established (Gibson et al. 2009), and wheel-running and feeding/drinking behaviors can be easily monitored. Less clear are the changes at the molecular level as a result of aging. Most studies that have investigated clock gene expression during aging were typically limited to a small set of genes (Banks et al. 2016; Kolker et al. 2003; Wyse and Coogan 2010), and it was not always clear due to constraint in temporal resolution whether the overall amplitude of the cellular oscillator was altered. Complementing the circadian reporter monitoring (e.g., PER2::Luc) (Yoo et al. 2004), mRNA and protein expression of core clock genes and canonical outputs such as *Dbp* should ideally be determined over the circadian cycle. A number of metabolic parameters can be investigated over the circadian cycle (He et al. 2016), such as blood glucose, oxygen consumption, and respiratory quotient.

A central metabolic process at the interface of aging, metabolism and circadian clock and thus critical for circadian therapeutic efficacy is the age-related decline in mitochondrial function and dynamism (Merksamer et al. 2013; Nguyen et al. 2013; Amara et al. 2007; Sastre et al. 1996). ROS produced by OXPHOS causes oxidative stress and damage to mitochondrial components. To minimize the damage, redox-sensitive transcription factors including NRF2 are activated to stimulate expression of antioxidant molecules (Scheele et al. 2009; Gorrini et al. 2013). Genes encoding several key factors have been found to harbor E-box and RORE elements recognized by CLOCK/BMAL1 and REV-ERBs and RORs respectively (Jacobi et al. 2015; Koike et al. 2012; Takeda et al. 2014). Furthermore, hepatic *Bmal1* knockout provokes reduction of mitochondrial mass and deterioration of mitochondrial respiration capacity, whereas hepatic overexpression of *Bmal1*

improved insulin sensitivity and glucose tolerance (Jacobi et al. 2015). These results suggest an integrated mechanism via which enhancement of circadian clock function serves to retard age-associated decline in mitochondrial function, including stimulation of anti-oxidant expression and function, mitochondrial respiration, and dynamism. In light of the comprehensive improvement of circadian and metabolic functions in mice treated with NOB, it will be of strong interest to investigate whether NOB can improve mitochondrial function and decelerate metabolic aging. Likewise, additional small molecules targeting RORs, REV-ERBs, and other clock components with reported metabolic benefits in vivo can also be tested (He and Chen 2016; Kojetin and Burris 2014; Chang et al. 2012). Given that certain compounds appeared to dampen circadian rhythm amplitude, contrary to the effect of NOB, long-term aging studies may provide important insight into respective benefits and side effects of these differentially-acting clock modulating molecules. It is possible that both directions of clock manipulation, as long as within a certain functional range, may induce beneficial metabolic and physiological improvement via distinct mechanisms (He et al. 2016; Solt et al. 2012).

11.7 Concluding Remarks

The efforts to exploit the clocks to decelerate metabolic and physiological deterioration and to promote healthy aging are only beginning, yet with great promise. On the one hand, the growing list of clock-modulating compounds with in vivo efficacies constitute an excellent toolkit to investigate their potential as anti-aging therapeutics. On the other hand, evidence is accumulating that CR, as well as lifestyle manipulations such as TRF and exercise known to promote healthy aging, significantly modulates the circadian clock (Lopez-Otin et al. 2013; Lopez-Otin et al. 2016; Longo and Panda 2016; Tevy et al. 2013). Many small molecules or drugs have been shown to extend lifespan and healthspan, including those deliberately designed to mimic CR and other manipulations (Lopez-Otin et al. 2016; Okada-Iwabu et al. 2013), and it would be interesting to characterize their circadian clock effects and potentially delineate causal mechanisms. Together, both approaches can synergize to decipher circadian and metabolic regulatory pathways ideally suited as anti-aging therapeutic targets. As illustrated by studies with NOB, it is possible that efficacious and nontoxic circadian therapeutics will exert a systemic and balanced effect over an extended period, potentially leading to improved lifespan and healthspan.

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Chapter 12
The Possible Role of Epigenetics in the Memory Impairment Elicited by Circadian Rhythm Disruption

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Abstract Memories are the glue of one's existence; unfortunately sometimes memories are more transitory then we would like. Aging and various disease states can induce memory impairments, but for the most part the mechanisms for these memory impairments are largely unknown. Circadian rhythms increase an organism's biological fitness by synchronizing its physiology and behaviour to their environment. Sometimes circadian rhythms become desynchronized from the environment and this circadian misalignment elicits memory impairments in both humans and rodents. Circadian rhythm dysfunction and memory impairments are hallmarks of both aging and chronic shiftwork, therefore it is pertinent to untangle the nature of the relationship between these processes. Epigenetics allow one's environment to influence gene expression without changing the genome itself. The plasticity of both memory and circadian rhythms are mediated in part by epigenetic modifications. While epigenetics is necessary for both circadian rhythm generation and memory, very little is known about how circadian rhythm disruption affects the epigenome. Epigenetics will be discussed as a mediator between circadian rhythms and memory in conditions where circadian rhythms are in or out of synch with the environment.

Keywords Circadian Rhythm Disruption · Epigentics · Memory · Aging · Age Related Cognitive Decline · Circadian Misalignment

Circadian rhythms are ingenious adaptations that allow organisms to be at their best at times when it is most likely to be beneficial. This is achieved by synchronizing our internal clock with the environmental clock. But, what happens when the time represented by the internal and environmental clocks is different? This lack of sync between our external environment and internal pacemaker is more pronounced in our society today than ever before. Recently, circadian rhythm disruption has been

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associated with various diseases, and even occurs in natural aging. Circadian rhythm disruption is a classic example of how one's environment can have negative effects on health. The mechanism's for the harmful effects of circadian rhythm disruption on the brain and body are largely yet to be explored. Epigenetics provides an avenue for how the environment can affect gene expression without changing DNA itself. Epigenetics are involved in the generation and synchronization of circadian rhythms but little is known about how this mechanism is affected by circadian rhythm disruption. The following chapter will briefly introduce the concept of epigenetics and their involvement with circadian rhythms. We will then discuss how memory is influenced by circadian rhythms and suggest that epigenetics is integral in this relationship when rhythms are both entrained and desynchronized from the environment.

12.1 Epigenetic Modifications

Relatively recently it was discovered that phenotype is not a static construct that is exclusively dictated by DNA, but instead there is an epigenome that influences gene expression without affecting the underlying DNA sequence (Goldberg et al. 2007; Kouzarides 2007). In the current paper epigenetics refers to any modification that affects gene expression and not just those that are heritable (Benayoun et al. 2015). The epigenome can vary stochastically, or can be manipulated by ones environment (Feil and Fraga 2012; Gallou-kabani and Junien 2007). Thus, the changes in the epigenome are not global but instead are tissue specific depending on the specific environmental exposure (Feil and Fraga 2012; Gallou-kabani and Junien 2007). These changes can be transient, long lasting, and in some instances heritable (Goldberg et al. 2007; Ledón-Rettig et al. 2012). While the epigenome is most malleable during gestation, it can also change during adulthood (Korkmaz et al. 2009; Feil and Fraga 2012).

Epigenetic modifications can be broadly classified into two categories, those that affect chromatin or DNA. Chromatin consists of DNA wrapped around histone proteins so that the genome can be housed in cell nuclei (Benayoun et al. 2015; Goldberg et al. 2007; Kouzarides 2007; Bernstein and Allis 2005). The first type of epigenetic modification involves histone manipulations that either suppress or promote gene expression by compacting (heterochromatin) or elongating (euchromatin) chromatin, respectively (Goldberg et al. 2007; Kouzarides 2007). The binding of various enzymes changes the positional arrangement of the histones. Although the effects of these enzymes can vary depending on the current conditions, they generally activate (acetylation, phosphorylation) or repress (methylation, sumoylation, deacetylation, deamination, and proline isomerization) transcription (Kouzarides 2007). Whereas, ubiquitination and methylation can do either depending on the circumstance (Kouzarides 2007).

The other primary form of epigenetic modification, DNA methylation, involves the repression of transcription via the binding of methyl groups to DNA (Haus and

Smolensky 2013; Korkmaz et al. 2009; Goldberg et al. 2007). Methyl groups typically bind to DNA in promoter regions that are rich in cytosine bases that are bound to guanine with a phosphodiester bond (CpG islands). Transcription is impeded when these typically unmethylated CpG islands become blocked by the binding of methyl groups (Korkmaz et al. 2009; Goldberg et al. 2007; Benayoun et al. 2015).

12.2 Circadian Rhythms

Circadian rhythms are endogenously generated oscillations in physiology and behaviour that are found in virtually all organisms ranging from bacteria to vertebrates (Young and Kay 2001). The sleep-wake cycle, activity-rest cycle, hormone secretion, body temperature, and metabolism are all examples of circadian rhythms (Barnard and Nolan 2008; Moore 1999). Environmental cues (zeitgebers) synchronize circadian rhythms with the environment so that organisms are able to anticipate daily events and adjust their physiology and/or behaviour accordingly to better take advantage of their environment (Merrow et al. 2005). Light is the strongest zeitgeber for circadian rhythms, but food, exercise, social interaction, and learning can entrain circadian rhythms in certain circumstances (Merrow et al. 2005; Krishnan and Lyons 2015; Ralph et al. 2013).

Circadian rhythms are an ideal system to study the link between genes and behaviour as they are generated at the cellular level by autoregulatory transcription/posttranscription/translation/post-translational feedback loops containing a concert of genes (clock genes) (Okamura 2004; Reppert and Weaver 2001). In short, CLOCK and BMAL1 form heterodimers that activate the transcription of CRY and PER homologues which then dimerize and move into the nucleus to inhibit the transcription of CLOCK and BMAL1 (Reppert and Weaver 2001; Okamura 2004). Another regulatory loop influences the core loop by promoting or suppressing transcription of BMAL1 via the binding of RORAα and REV-ERBα, respectively, to ROR elements in the BMAL1 promoter region (Reppert and Weaver 2001; Okamura 2004). Post-translational modifications are involved in the fine-tuning of these feedback loops (Ko and Takahashi 2006).

Amazingly, depending on the tissue and cell type, the expression of up to 30% of transcripts display diurnal variations (Aguilar-Arnal and Sassone-Corsi 2014; Panda et al. 2002; Storch et al. 2002; Duffield et al. 2002). Although, in vertebrates molecular oscillations are occurring in virtually all tissues, a master clock located in the anterior hypothalamus, the suprachiasmatic nucleus (SCN), is necessary for the generation of most rhythms and more importantly the synchronization of the peripheral brain and body clocks (Dibner et al. 2010; Reppert and Weaver 2001; Antle and Silver 2009). It is thought that the SCN uses neuropeptides, direct neuronal efferents, and hormones to coordinate rhythms in other tissues (Mohawk et al. 2012; Guilding and Piggins 2007; Antle and Silver 2009). SCN activity and dependent rhythms persist in the absence of zeitgebers, whereas the oscillations and

synchronization of most peripheral clocks is dependent on the SCN (Guilding and Piggins 2007; Yoo et al. 2004; Antle and Silver 2009; Mohawk et al. 2012).

12.2.1 Epigenetic Involvement

As the environment entrains circadian rhythms, it is not surprising that epigenetics are involved in both the generation and regulation of circadian rhythms. Some of the clock genes have epigenetic functions, while the functioning of others is effected by epigenetic modifications. The first evidence of an epigenetic involvement in the circadian clock was that in addition to acetylating BMAL1, CLOCK can also activate transcription of its target genes by adding acetyl groups to histones [histone acetyltransferase enzyme (HAT)] in a rhythmic fashion (Doi et al. 2006; Hirayama et al. 2007; Bellet and Sassone-Corsi 2010). Similarly, mixed-lineage leukemia 1 (MLL1) is a methyltransferase (adds methyl groups to histones) that is necessary for the oscillation of all the core clock genes as it enhances the transcriptional activation of BMAL1:CLOCK heterodimers (Katada and Sassone-Corsi 2010).

Some of the sirtuin enzymes, which are involved in inflammation, aging, and metabolism, are directly involved in the circadian clock (Orozco-Solis and Sassone-Corsi 2014; Jenwitheesuk et al. 2014). Sirtuin1 (SIRT1), is a metabolic sensor involved in the regulation of cellular energy that is expressed in areas of the brain such as the hippocampus, cortex, hypothalamus, and cerebellum (Zakhary et al. 2010). SIRT1 is thought to influence a wide array of functions ranging from neurogenesis, synaptic plasticity, DNA repair, cell cycle arrest, cell survival, gluconeogenesis, lipid metabolism, insulin sensitivity, and protection against brain pathology associated with Alzheimer's disease (Orozco-Solis and Sassone-Corsi 2014; Jenwitheesuk et al. 2014; Gao et al. 2010; Michán et al. 2010).

Recently it was discovered that SIRT1 is directly involved in the core circadian clock. In contrast to CLOCK, SIRT1, downregulates CLOCK:BMAL1 mediated transcription by rhythmically deacetylating BMAL1 [histone deacetylase enzyme (HDAC)] (Asher et al. 2008; Nakahata et al. 2008). SIRT1 can oscillate in some tissues, such as the SCN (Chang and Guarente 2013) and hippocampus (Rawashdeh et al. 2014), however this diurnal expression is a byproduct of the rhythmic activity of its cofactor and activity regulator nicotinamide adenine dinucleotide (NAD^+) (Eckel-Mahan and Sassone-Corsi 2013; Orozco-Solis and Sassone-Corsi 2014; Jenwitheesuk et al. 2014).

Besides being involved in the generation of circadian rhythms, epigenetic activity can be influenced by circadian rhythms. Some have theorized that rhythmic epigenetic interactions with the core clock contribute to the high degree of tissue specific variability in circadian expressed genes (Masri and Sassone-Corsi 2010). Histone modifiers oscillate in the SCN (Qureshi and Mehler 2014) and hippocampus (Rawashdeh et al. 2014). Similarly, DNA methylation and associated

molecules oscillate in various tissues and can even be manipulated by zeitgebers (Qureshi and Mehler 2014).

Chronotype is an example of how the interaction between epigenetics and circadian rhythms is a synergistic one. Chronotype is a term used to describe the entrainment of one's circadian rhythms to the environment with the idea that some individual's clocks are early and some are late (Barclay et al. 2013; Roenneberg et al. 2004). Chronotype contributes to ones preferred times of activity and sleep (Barclay et al. 2013; Roenneberg et al. 2004). As it is thought a combination of genetics and one's environment determines chronotype, epigenetics is likely involved in determining chronotype (Barclay et al. 2013; Roenneberg et al. 2004). Recent rodent and human data suggests that the epigenome is involved in entrainment to the environment. Stochastic variations in the epigenome has been suggested as a possible explanation for differing chronotypes in inbred mice that have very similar DNA and identical housing conditions (Barclay et al. 2013). Entrainment to a new photoperiod, or seasonal adaptation to shorter photoperiod requires DNA methylation in the SCN and hypothalamus, respectively (Azzi et al. 2014; Stevenson and Prendergast 2013). Several studies in humans also suggest that DNA methylation might contribute to chronotype (Bollati et al. 2010; Milagro et al. 2012).

In summary epigenetic mechanisms are integral to circadian rhythm generation at the molecular and tissue level. The finding that epigenetics are involved in the entrainment of circadian rhythms indicates that they could be a possible mechanism for the deleterious effects of circadian rhythm disruption.

12.3 Circadian Rhythm Disruption

Major problems that affect virtually all aspects of physiology arise when circadian rhythms are unsynchronized from the environment (Zelinski et al. 2014a; Kuhn 2001). Realities of life today such as exposure to artificial light at night, jet lag, and shift work are particularly challenging for circadian rhythms (Zelinski et al. 2014a; Haus and Smolensky 2013; Banks et al. 2016). 15% of the global work force in developed countries are shift workers, not to mention that working longer hours and at times outside of the normal day shift is increasing in the United states (Alterman et al. 2013; Krishnan and Lyons 2015; Wright et al. 2013). Under these conditions circadian rhythms become misaligned from the cues entraining them. Chronic circadian rhythm misalignment has been associated with cognitive impairments, heart disease, metabolic syndrome, shortened lifespan, and cancer (Zelinski et al. 2014a; Banks et al. 2016; Haus and Smolensky 2006).

Animal models of circadian rhythm disruption are particularly useful because circadian rhythms can be directly manipulated, there is no self-selection bias that is evident in human clinical populations, and the effects of circadian rhythm disruption on many different tissues such as the brain can be investigated. There are a variety of methods to induce circadian rhythm disruption, such as lesioning the

SCN, transgenic manipulations of clock genes, constant exposure to light or dim light, forced activity or feeding during the rest phase of the light dark cycle, and changing the light dark cycle (for review see Zelinski et al. 2014a; Deibel et al. 2015; Krishnan and Lyons 2015; Smarr et al. 2014). Our lab has created a rat circadian rhythm disruption paradigm that induces circadian rhythm misalignment by changing the light-dark cycle for a brief or lengthy period of time (Zelinski et al. 2014a; Deibel et al. 2015). All of these different paradigms have advantages and disadvantages in terms of pinning down possible mechanisms, or in the type of circadian rhythm disruption elicited. For example, one type of circadian rhythm disruption could cause rhythms to no longer oscillate in a circadian manner, or instead, oscillations could still be circadian but no longer synchronized to the light dark cycle (Zelinski et al. 2014a; Deibel et al. 2015; Smarr et al. 2014; Krishnan and Lyons 2015). The hallmark of shiftwork in humans, which is embodied in our and other animal models, is an inability to entrain to the aberrant work schedule and thus circadian rhythms are out of phase with the new light dark cycle (Haus and Smolensky 2006; Castanon-Cervantes et al. 2010; Gibson et al. 2010; Loh et al. 2010; Sei et al. 1992; Van Dycke et al. 2015; Fonken et al. 2010; Salgado-Delgado et al. 2008).

Circadian rhythm disruption is thought to be a mechanism responsible for the harmful effects of shiftwork on the brain and the body, however the nature of its specific effects is not fully known. The following sections will focus on how circadian rhythms are involved in learning and memory under entrained conditions and in situations that induce circadian rhythm disruption, such as shiftwork and natural aging. Epigenetics will be portrayed as a possible mechanism for how circadian rhythm disruption affects memory.

12.3.1 Learning and Memory

Circadian rhythms and memory are inextricably linked. The time that something occurs or is learned is an important part of both memory acquisition and retrieval. The animal literature is rich with observations of how these two processes interact. We will review these data and then discuss how epigenetics is/or might be involved in these processes.

12.3.1.1 Time-Place-Learning

Stemming from observations in the wild that organisms visit locations at times of day when it is most beneficial for them to forage, animal's ability to encode time has been studied extensively in the lab (Daan and Koene 1981; Thorpe and Wilkie 2006; Moore-Ede et al. 1982). In daily time-place-learning studies, animals must learn to go to certain locations at a specific time of day to receive a reward or avoid an aversive stimulus (Thorpe and Wilkie 2006; Mulder et al. 2013a). In some of

these paradigms, rats' use a circadian timer to associate a specific time of day with a location (Deibel and Thorpe 2012; Mistlberger et al. 1996; Van der Zee et al. 2008; Thorpe and Wilkie 2007). However, the story in rats is a complex one as performance in time-place learning tasks might vary depending on the type of task used, or the strain of rat used (Thorpe et al. 2012; Deibel et al. 2014b). Mechanistically, time-place-learning requires *Cry 1 & 2* (Van der Zee et al. 2008), but not *Per 1 & 2* genes (Mulder et al. 2013b), nor does it require an intact SCN (Mistlberger et al. 1996). These data suggest that a peripheral circadian oscillator is likely mediating performance, but it remains to be seen the exact nature of the mechanisms underlying time-place learning.

12.3.1.2 Time Stamping and Circadian Variations in Cognition

The time-stamping phenomenon is another classic example of how time is involved in memory. As pioneered by our research group, some rodents only display memory for a context when their memory is tested at the same time that learning occurred. This is true for hamsters' memory of both aversive and appetitive contexts (Cain et al. 2004a; Ralph et al. 2002). Similar to the circadian modulation of time-place-learning, the relationship between circadian rhythms and time-stamping is complex as only some rat strains show evidence of time-stamping (McDonald et al. 2002; Cain et al. 2004c). Similarly in hamsters, time-stamping also does not require an intact SCN (Ko et al. 2003; Cain et al. 2012). As with time-place learning, a peripheral oscillator appears to be modulating the temporal gating of context memory inherent to time stamping. Ralph et al. (2013) provided evidence suggesting that there is a context-entrainable oscillator mediating performance, however the locus of said oscillator is unknown.

An extension of time stamping is the finding that some rodents learn better at different phases of the circadian cycle. In rodents, acquisition and/or retention of an operant task, or hippocampal dependent tasks such as contextual fear conditioning, spatial radial arm maze task, novel object location task, and spatial learning in the Morris water task (MWT) is better during the night time (Hauber and Bareiß 2001; Valentinuzzi et al. 2001, 2004; Gritton et al. 2012; Takahashi et al. 2013). Amazingly, in the MWT, while there were no time-of-day effects in acquisition on a recent probe trial performance, spatial memory in a probe trial two weeks after the cessation of training was stronger for rats that were trained during the night time (Gritton et al. 2012). In contrast, in mice, some studies suggests that recall for various paradigms such as contextual fear conditioning, fear conditioning to tone, and spatial working memory is better during the day-time (Chaudhury and Colwell 2002; Eckel-Mahan et al. 2008; Rawashdeh et al. 2014).

12.3.1.3 Circadian Misalignment

Time-place-learning, time stamping, and circadian variations in cognition are examples of how circadian rhythms influence learning and memory under entrained conditions, however as discussed earlier, there are many circumstances in which circadian rhythms are not entrained. Memory impairments have been associated with long periods of time working shift work or in the air travel industry (Rouch et al. 2005; Marquié et al. 2014; Cho 2001; Cho et al. 2000).

Many laboratories have investigated the effects of circadian rhythm disruption on learning and memory in rodents. It was first discovered that memory retention in active and passive avoidance tasks was impaired by changing the light dark cycle (Davies et al. 1974; Fekete et al. 1986, 1985; Tapp and Holloway 1981; Stone et al. 1992). However, these studies possessed some caveats. First, theses studies used strains of rats that display time stamping, thus it was unclear if the memory impairment was due to circadian rhythm disruption, or training and testing occurring at different times of day. Second, their pattern of impairments suggested that the animals were not re-entrained during the memory test, which could mean that impairments were due to state-dependency differences between training and testing. Finally and perhaps most important it was unclear if other types of memory, such as episodic memory would be effected.

The hippocampus is thought to mediate episodic memory in humans and thus has been extensively modeled in rodents (Tulving 1993; Tulving et al. 1998). The Morris water-task (MWT) is the gold standard measure of hippocampal dependent memory in rodents (McDonald et al. 2004). In the standard version, rats learn to navigate to a hidden platform in a large pool of water over eight trials administered each day for four or five consecutive days (McDonald et al. 2004). It is thought that rats use an internal compass (path integrator) in conjunction with the arrangement of distal cues in the room to navigate to the platform (McDonald et al. 2004). Memory is then tested in a probe trial that involves returning the animal to the pool without the platform present with the idea that an animal with a spatial memory will spend more time in the area of the pool that contained the platform during training (McDonald et al. 2004).

Given that circadian rhythms and hippocampal dependent memory both decline with age, we hypothesized that circadian rhythm disruption would impair hippocampal dependent memory. As outlined in Table 12.1, our lab has created a rat circadian rhythm disruption paradigm that induces circadian rhythm misalignment by changing the light-dark cycle for a brief or lengthy period of time (Zelinski et al. 2014a; Deibel et al. 2015). In the acute paradigm, the light onset or offset occurs three hours earlier (3 h phase advance) for six consecutive days (Devan et al. 2001; Craig and McDonald 2008; Zelinski et al. 2014b). It takes approximately 20 days to re-entrain after the acute paradigm (Devan et al. 2001; Craig and McDonald 2008). In our chronic 64 day paradigm, there are four cycles of the acute paradigm, with each cycle separated by 10 days of re-entrainment to a normal 12:12 LD cycle (Craig and McDonald 2008; Deibel et al. 2014a; Zelinski et al. 2013). This paradigm continuously challenges the circadian system, as rhythms are not able to

entrain during these brief 10 day periods (Craig and McDonald 2008). Re-entrainment had not occurred 20 days after the cessation of phase advances in the chronic paradigm (Craig and McDonald 2008).

A T cycle is a non 24 h light dark cycle, and our 3 h phase advance is concurrent with a T21 cycle with either 43% (L9:D12) or 57% (L12:D9) light depending on if the 3-h are removed from the light or dark phase of the cycle (Devan et al. 2001; Craig and McDonald 2008; Rota et al. 2016). During the phase advances, rhythmicity is maintained and the period (time it take to complete one cycle of a rhythm) does not change (Rota et al. 2016). Rather this paradigm appears to induce free-running conditions, which means that during the phase advances, rhythms are very out-of phase with the light dark cycle (Devan et al. 2001; Craig and McDonald 2008; Zelinski et al. 2013, 2014b; Rota et al. 2016). This is evidenced by much more activity and much less sleep during the normal resting period (Rota et al. 2016; Craig and McDonald 2008; Devan et al. 2001; Zelinski et al. 2013, 2014b).

Table 12.1 An example of the light schedule for our circadian rhythm disruption paradigms

Day	Lights on
1	01:30
2	22:30
3	19:30
4	16:30
5	13:30
6	10:30
7–16	Re-entrainment 07:30
17	04:30
18	01:30
19	22:30
20	19:30
21	16:30
22	13:30
23–32	Re-entrainment 10:30
33	07:30
34	04:30
35	01:30
36	22:30
37	19:30
38	16:30
39–48	13:30
49	10:30
50	07:30
51	04:30
52	01:30
53	22:30
54	19:30
55–64	16:30

Day 16 is the cessation of the acute paradigm, whereas day 64 is the cessation of the chronic paradigm. In this example, lights on was advanced by three hours, so in the T21 cycle, there was 57% light and 43% dark (L12:D9)

Rats that received five days of training in the standard version of the MWT during the acute paradigm were able to learn the location of the platform during training as evidenced by decreasing latencies and path lengths to the platform (Devan et al. 2001). However, as shown in Fig. 12.1, when memory retention was tested in probe tests seven and 17 days after the end of acquisition, the circadian rhythm disrupted rats did not show evidence of remembering the platform location (Devan et al. 2001). In contrast to the interpretation from the early demonstrations in active and passive avoidance tasks, we suggested that the retention deficit appeared to be due to a failure to consolidate the memory, as learning was not affected (Devan et al. 2001).

While consolidation of a hippocampal dependent memory was impaired if acquisition occurred during circadian rhythm disruption, it was unclear if circadian rhythm disruption could have anterograde and retrograde amnestic effects on memory. In the anterograde assessment we used a more sensitive MWT variant that could tease apart distributed and massed training deficits (Craig and McDonald 2008). Training was started 10 days after the end of the phase advances in both the acute and chronic paradigms (Craig and McDonald 2008). The chronically circadian disrupted rats had impaired: distributed acquisition, rapid acquisition of a novel platform location, and impaired retention of either of these spatial locations (Craig and McDonald 2008). The rats that experienced acute circadian rhythm disruption and then training were indistinguishable from controls, which suggests the consolidation deficit observed previously in the acute paradigm is a result of

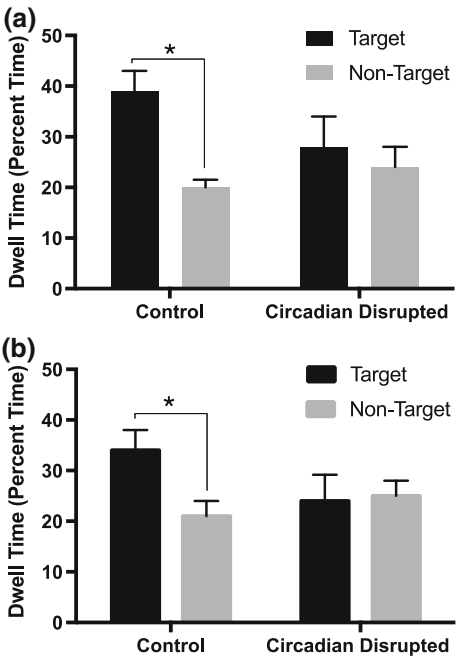

Fig. 12.1 Male rats that received training in the standard version of the MWT during the phase advance period of the acute paradigm, had impaired retention of the spatial memory in 60 s probe trials seven (**a**) and 17 (**b**) days after training. "Target" refers to the percentage of time spent in the quadrant of the pool that contained the platform during training, whereas "Non-target" is the average percentage of time spent in the remaining three quadrants (figure modified from Devan et al. 2001 with permission)

phase advances occurring during learning (Craig and McDonald 2008; Devan et al. 2001). That being said, chronic circadian rhythm disruption has anterograde amnestic effects on hippocampal dependent memory that affect both acquisition and retention of the information.

The next series of experiments from our lab determined if acute or chronic circadian rhythm disruption has a retrograde amnestic effect on hippocampal dependent memory by exposing the animals to circadian rhythm disruption after memory acquisition. Interestingly, both acute and chronic circadian rhythm disruption induced retrograde amnesia in the MWT (Zelinski et al. 2013, 2014b). These studies also investigated if sex or access to a running wheel influenced the memory impairment elicited by circadian rhythm disruption. Two main findings emerged: (1) Memory impairments were more severe in males. (2) The memory impairment was more pronounced for animals without wheels (Zelinski et al. 2013, 2014b). In tandem with our previous studies, these data as a whole suggest that circadian rhythm disruption has more severe effects on hippocampal dependent memory when acquisition occurs during or before circadian rhythm disruption.

While the studies mentioned above clearly suggest that the hippocampus is sensitive to circadian rhythm disruption as assessed via the MWT, what about other tasks that assess hippocampal function? Both acute and chronically circadian disrupted rats were unimpaired in both tone and contextual fear conditioning (Craig and McDonald 2008). The fear-conditioning paradigm used, likely requires minimal hippocampal involvement, however it remains a possibility that fear memory could be susceptible to circadian rhythm disruption if acquisition occurs during or before disruption (Craig and McDonald 2008). For example, in mice, single phase advances right before or after contextual fear conditioning impair retention but not acquisition of the fear memory (Loh et al. 2010). We also assessed the effects of our chronic circadian rhythm disruption paradigm in a version of fear conditioning that relies more on the hippocampus because it involves the discrimination of several contexts (Antoniadis and McDonald 2000). Remarkably, rats that experienced chronic circadian rhythm disruption actually performed better in a measure of this task (Deibel et al. 2014a). Similarly, mice that experienced multiple phase shifts were indistinguishable from controls and better than animals that received a single phase shift in the recall of contextual fear conditioning (Loh et al. 2010). As will be discussed below, it is possible that a potentiated stress response from chronic phase shifting is resulting in facilitation of the fear memory in these instances (Deibel et al. 2014a).

While the fear conditioning tasks mentioned above require the amygdala, the hippocampus is also a key player in fear conditioning (Antoniadis and McDonald 2000). Thus, we wanted to investigate the effects of our circadian rhythm disruption paradigms on other behaviours that do not involve the hippocampus. We have developed a stimulus-response visual discrimination task in the radial-arm-maze that requires the dorsal striatum, but not the hippocampus (McDonald and Hong 2004; McDonald and White 1993). This task is particularly useful because cognitive flexibility, which is reliant on the prefrontal cortex can be assessed by reversing the reward contingencies (Zelinski et al. 2013, 2014b). Performance in the

visual discrimination task and the cognitive flexibility reversal of the reward contingencies was impaired in the chronic, but not acute circadian rhythm disruption paradigm (Zelinski et al. 2013, 2014b). As with the MWT data, males and animals without wheels also performed poorer in these tasks. These data as a whole suggest that circadian rhythm disruption does not just affect hippocampal dependent memory, but can affect behaviours reliant on the dorsal striatum and prefrontal cortex. It is also possible that this retrograde amnesia effect for both hippocampal and non-hippocampal-dependent tasks is due to a change in the hierarchical interaction of various brain circuits that is a result of impaired hippocampal functioning induced by circadian rhythm disruption. Readers are encouraged to see a recent theoretical paper from our group on the interaction of various neural circuits in anterograde and retrograde amnesia (Lee et al. 2016).

As with the time-place-learning and time stamping, the effect of circadian rhythm disruption on memory is a complex one that depends on sex, when the circadian rhythm disruption occurs, task used, and whether the animals had access to a wheel. It is unclear why some tasks are more susceptible to the effects of circadian rhythm disruption, nor why females might be more protected than males under some conditions. However, undeniably, data from our lab, and other labs in various rodent species (Fekete et al. 1985, 1986; Loh et al. 2010, 2015; Gibson et al. 2010; Ruby et al. 2008; Fujioka et al. 2011; Fernandez et al. 2014) unanimously suggest that circadian rhythm disruption can impair memory.

12.3.1.4 Aging

Aspects of circadian rhythms such as free-running period, amplitude, rhythm fragmentation, and ability to entrain to a new photoperiod are affected by aging (Gutman et al. 2011; Morin 1988; Penev et al. 1995; Pittendrigh and Daan 1974; Possidente et al. 1995; Valentinuzzi et al. 1997; Wax 1975; Welsh et al. 1986; Scarbrough et al. 1997; Pandi-perumal et al. 2002). Circadian rhythm degradation that occurs during aging does not appear to be due to SCN cell loss, but rather a loss of synchronization among cells which is likely due to reduced efficacy of SCN neurotransmission (Farajnia et al. 2013; Palomba et al. 2008; Kalló et al. 2004; Kawakami et al. 1997).

One of the hallmarks of aging is cognitive decline, which is often mild, but can be severe in dementias such as Alzheimer's disease (AD) (Penner et al. 2010; Petersen et al. 1999). The ability to form and recall episodic memories, which are memories for the time and place of personally experienced event, is compromised in normal aging and in AD (Souchay et al. 2000; Leube et al. 2008; Storandt 2008; Tulving 1993; Tulving et al. 1998). As with the degradation of the circadian system that occurs with aging, normal aging is not associated with massive degeneration in areas of the brain, such as the hippocampus that are involved in learning and memory (Keuker et al. 2003; Rapp and Gallagher 1996; West et al. 1994; Burke and Barnes 2006). However, as will be discussed below various mechanisms

involved in hippocampal synaptic plasticity are negatively impacted by aging (Penner et al. 2010).

An intriguing hypothesis is that the circadian rhythm disruption that occurs during aging is contributing to the age-associated cognitive decline (Deibel et al. 2015; Popa-Wagner et al. 2015; Krishnan and Lyons 2015; Coogan et al. 2013). As demonstrated in Fig. 12.2, our group, first demonstrated this by observing that aged hamsters with naturally occurring disrupted circadian rhythms had poorer hippocampal dependent memory than aged-matched hamsters with better circadian rhythms (Antoniadis et al. 2000). These findings have been extended to humans, as decreased circadian rhythm amplitude, altered phase of rhythms, or rhythm fragmentation in normal healthy aged individuals was associated with poorer executive function, higher incidences of mild cognitive impairments, and dementia (Tranah et al. 2011; Walsh et al. 2014; Schlosser Covell et al. 2012; Lim et al. 2012). This hypothesis is relatively unexplored, particularly in animal models, and is very appealing as improving circadian rhythmicity could partially rescue the age-associated cognitive decline (Deibel et al. 2015; Krishnan and Lyons 2015; Popa-Wagner et al. 2015; Coogan et al. 2013; Froy 2013). Some data in humans suggests that this might be possible, as improving circadian rhythmicity in older adults can improve cognition (Peck et al. 2004; Jean-Louis et al. 1998; Cardinali et al. 2012).

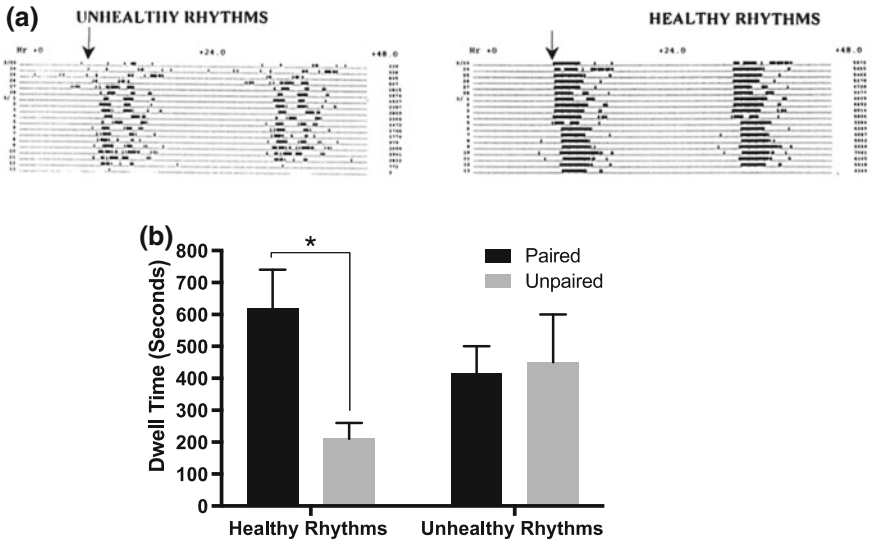

Fig. 12.2 Aged hamsters with naturally unhealthy circadian rhythms did not have a preference for an appetitive context, whereas aged matched animals with naturally healthy circadian rhythms did show a preference. **a** Representative actograms from a hamster with unhealthy rhythms and a hamster with healthy rhythms. **b** Dwell time in a preference test for a context associated with a running wheel (paired), and a context that was not associated with a running wheel (unpaired) (figure modified from Antoniadis et al. 2000 with permission)

As will be demonstrated in the following sections many of the possible mechanisms for memory impairments induced by circadian rhythm disruption due to misalignment or aging are likely similar. However, isolating the impact of circadian rhythm disruption on memory in the aged phenotype is very difficult, as aging affects virtually all aspects of physiology and behaviour. Nonetheless, the next section will discuss possible mechanisms for the circadian rhythm disruption induced memory impairment and how these mechanisms are affected by aging will also be briefly explored.

12.4 Mechanisms for Circadian Rhythm Induced Memory Impairment

The proposed neural correlate of memory, long term potentiation (LTP), is a persistent strengthening of neuronal connections that occurs with repeated activations of those connections (Bliss and Lømo 1973; Penner et al. 2010). At the molecular level, hippocampal dependent memory is thought to involve activation of the cAMP/$Ca2^{+}$ response element (CRE) transcription pathway involving sequential activation of cyclic adenosine monophosphate (cAMP), mitogen activated protein kinase (MAPK), and cAMP responsive element binding protein (CREB) (Eckel-Mahan et al. 2008). Circadian rhythm disruption is affecting hippocampal plasticity, and there is a myriad of possible ways in which it might do so, however how these mechanisms interact in situations of circadian rhythm disruption is largely unknown. For in depth discussions, readers are encouraged to consult reviews from our and other labs (Zelinski et al. 2014a; Deibel et al. 2015; Smarr et al. 2014; Krishnan and Lyons 2015; Eckel-Mahan and Storm 2009). We will discuss some of the most likely possibilities below.

12.4.1 Clock Genes

Hippocampal clock genes are likely involved in the influence of circadian rhythms on memory. Clock genes display circadian oscillations in the hippocampus (Jilg et al. 2010; Eckel-Mahan et al. 2008; Phan et al. 2011; Wang et al. 2009; Feillet et al. 2008; Wakamatsu et al. 2001; Rawashdeh et al. 2014). These oscillations appear to be dependent on the SCN (Lamont et al. 2005), although *Per2* continued to produce rhythms for days in a hippocampal slice preparation, suggesting that the hippocampus could be a semi-autonomous oscillator (Wang et al. 2009). Molecules mentioned above that are involved in hippocampal plasticity, such as cAMP, Ca2 stimulated adenylyl cyclases, Ras, and MAPK, are expressed in a circadian manner in the hippocampus (Eckel-Mahan et al. 2008; Phan et al. 2011). Interestingly a recent, study extended these findings to other molecules involved in memory that

oscillate in the hippocampus: *Arc, Bdnf*, *CBP*, and *P300* (Peixoto et al. 2015). Given that many of the molecules involved in memory oscillate, LTP facilitation and decay also display circadian rhythms in rodents with greater magnitude of enhancement and slower decay during the dark phase of the light cycle (Chaudhury et al. 2005; Barnes et al. 1977).

Clock genes have been directly linked to the molecules involved in the hippocampal signal transduction pathway as decreased amounts, and abolition of circadian oscillations of phosphorylated CREB are found in *Per2* (Wang et al. 2009) and *Per1* (Rawashdeh et al. 2014) mutant mice, respectively. Similarly, oscillations of MAPK and cAMP are abolished in *Bmal1* mutant mice (Wardlaw et al. 2014). Not surprisingly, mutant mice with deletions of various clock genes such as *Per1−/−* (Rawashdeh et al. 2014), *Per2−/−* (Wang et al. 2009), and *Bmal1 −/−* (Wardlaw et al. 2014) have impaired LTP. Mice with mutations of the core clock genes are also impaired in a variety of learning and memory tasks thought to rely on the hippocampus (*Per1−/−*: (Jilg et al. 2010; Rawashdeh et al. 2014); *Cry1 −/− Cry2−/−*: (Van der Zee et al. 2008); *Per2−/−*: (Wang et al. 2009); *Bmal1−/−*: (Kondratova et al. 2010; Wardlaw et al. 2014); *Clk*: (Kondratova et al. 2010).

The day time variations in memory acquisition and recall mentioned above are likely associated with these circadian variations in the hippocampal signal transduction pathway. Better day-time performance has been associated with the acrophase of MAPK (Eckel-Mahan et al. 2008) and this activity requires *Per1* (Rawashdeh et al. 2014). Conversely, better night time performance might be associated with increased LTP enhancement that occurs during the night time (Krishnan and Lyons 2015).

There is a central caveat in virtually of all of the studies mentioned above linking clock genes to the hippocampal signal transduction pathway. It is not known if the effects on hippocampal plasticity molecules are a result of global circadian rhythm arrhythmicity at the level of the SCN and/or altered hippocampal oscillations. SCN lesions impair retention of contextual fear conditioning and MWT in mice, which appears to be due to altered rhythms of the molecules involved in hippocampal plasticity (Phan et al. 2011). However, other studies in rats and hamsters fail to find an effect of SCN lesions in various types of memory (Stephan and Kovacevic 1978; Mistlberger et al. 1996; Fernandez et al. 2014). Several recent studies have contributed greatly to the suspected role of the SCN in the memory. First, as touched on above, Ralph and colleagues (Ralph et al. 2013) recently suggested that there might be a context-entrainable oscillator in which temporally gated memories drift to a new time without input (training), and this drift is dependent on the SCN. These data suggests that the contextual oscillator is working in concert with the SCN. Fernandez and colleagues (Fernandez et al. 2014), have recently investigated the role of the SCN in circadian rhythm induced memory impairments. In their hamster paradigm a phase advance, followed by a phase delay the next night produces circadian arrhythmicity and impairments in novel object recognition (Ruby et al. 2008; Fernandez et al. 2014). While SCN lesions did not impair novel object recognition, remarkably SCN lesions rescued the memory impairment elicited by the phase shifts (Fernandez et al. 2014). In accordance with this group's previous

finding that a GABA antagonist also rescues the same memory impairment, they are suggesting that the SCN has an inhibitory effect on memory that produces memory impairments under circadian rhythm disruption (Ruby et al. 2008; Fernandez et al. 2014). As will be discussed below there are a myriad of possible circadian outputs in addition to GABA that could be involved so the influence of circadian rhythms on memory may not be exclusively inhibitory. In conjunction with the findings from Ralph and colleagues (Ralph et al. 2013), an alternative explanation, is that the impairment is a result of misalignment between the SCN and a contextual oscillator that disappears when SCN inputs are abolished. These data suggest that as echoed throughout this chapter, circadian misalignment among rhythms or with rhythms and zeitgebers is likely more harmful than the abolition of circadian rhythms.

As with sleep and activity in our circadian disruption paradigm, during circadian misalignment hippocampal oscillations are likely out of phase with the light dark cycle, and possibly the phase relationship with the SCN is changed. Along these lines, in a very interesting recent paper that induced circadian rhythm misalignment in mice by restricted feeding during the daytime, the authors observed memory impairments, a major phase shift in hippocampal but not SCN clock gene oscillations, impaired LTP, and reductions in the total amount of CREB (Loh et al. 2015). Dim light at night has also been shown to alter the oscillations of some clock proteins in the SCN and hippocampus without affecting the activity rhythm (Bedrosian et al. 2013).

In addition to manipulations that affect circadian rhythmicity, clock gene oscillations in the SCN and peripheral oscillators, such as the hippocampus can also be abolished or dampened in aged rodents (Mattam and Jagota 2014; Weinert et al. 2001; Kolker et al. 2003; Wyse and Coogan 2010). Similarly, in AD brains, there are changes in the phase of *Per1*, *Per2,* and *Bmal1* oscillations in various brain oscillators (Cermakian et al. 2011). Clock genes also appear to be directly involved in aging, as *Bmal1* and *Clock* mutant mice have decreased lifespans, with *Bmal1* knockout mice also having an advanced aging phenotype (Kondratov et al. 2006; Dubrovsky et al. 2010). While data from clock gene manipulations, aging, and other environmental manipulations suggest that clock genes are clearly impacted by circadian rhythm disruption, hippocampal oscillations have yet to be assessed in paradigms that use light manipulation such as ours, and these studies could prove to be crucial in unearthing the mechanisms of circadian rhythm disruption induced memory impairments. Hippocampal specific clock gene mutants, or techniques like optogenetics or designer receptors exclusively activated by designer drugs (DREADDs) that can selectively active/deactivate neurons that express specific genes will also help answer this question.

12.4.2 Epigenetics

12.4.2.1 Clock Genes

Circadian rhythm disruption can affect the epigenome and has been suggested as a possible mechanism for the harmful effects of circadian rhythm disruption on health (Smarr et al. 2014; Zhu et al. 2011; Korkmaz et al. 2009). In the blood of shift-workers, *Clock* was hypomethylated (Zhu et al. 2011), whereas *Cry2* (Zhu et al. 2011) and the promoter region for a micro RNA (miRNA; miR-219) (Shi et al. 2013) involved in the free-running period were hypermethylated (Zhu et al. 2011). Long term shift work has also been associated with global reductions in methylation of some repetitive elements (Bollati et al. 2010). These data suggest that circadian misalignment or disruption induces epigenetic changes in genes that regulate circadian rhythms and as highlighted above, these changes could potentially change the period and or phase of circadian rhythms.

12.4.2.2 Cancer

As mentioned in the introduction, shiftwork has recently been identified as a possible carcinogen (Masri et al. 2015; Kochan and Kovalchuk 2015). Recent data from both shift workers and animal models of circadian rhythm disruption suggests that circadian rhythm disruption elicits epigenetic changes in genes that are involved in cancer. Coincidently, or perhaps not, as in the blood of shift workers, *Clock* and *Cry2* are also hypomethylated and hypermethylated, respectively, in the blood and tumors of breast cancer patients Hoffman et al. (2010a, b). Cytokines and imprinted genes that are involved in cancer are also differentially methylated in the blood of shift workers (Bollati et al. 2010; Jacobs et al. 2013).

In mice, light at night induced circadian rhythm disruption, leads to global DNA hypomethylation in breast cancer tumors, an effect that can be rescued by ameliorating the circadian rhythm induced attenuation of melatonin with exogenous melatonin (Schwimmer et al. 2014). Phase shifts of the light dark cycle in a similar manner to our paradigm, sped up the development of breast cancer tumors in a susceptible mouse strain (Van Dycke et al. 2015). This study adds to the findings observed by Schwimmer and colleagues (Schwimmer et al. 2014), by demonstrating that melatonin is not the only player in this story, as the mice in their study were melatonin deficient (Van Dycke et al. 2015). Instead circadian outputs such as corticosterone, sleep, and liver clock gene expression were altered, suggesting that there are many mechanisms involved (Van Dycke et al. 2015).

Our group has recently assessed the effects of our circadian rhythm disruption paradigms on large-scale miRNA and mRNA expression in mammary gland tissue from female Sprague Dawley rats. Both acute and chronic paradigms induced changes in many miRNAs involved in cancer and circadian rhythms that were dependent on the zeitgeber (ZT) time of tissue extraction (Kochan et al. 2015).

Remarkably, there were still changes in miRNA two weeks after the cessation of the chronic paradigm, an effect that was absent in the acutely shifted animals (Kochan et al. 2015). In terms of mRNA expression, genes involved in DNA repair were down regulated two weeks after the chronic paradigm in the animals that were sacrificed at ZT19 (Kochan et al. 2016). As a whole these data suggest that environmental circadian misalignment similar to that elicited by shift work induces immediate and/or long lasting changes in miRNA and mRNA expression in the mammary gland tissue of rats. To our knowledge these are the first studies to conduct large-scale miRNA and mRNA assessments in mammary gland tissue of rats exposed to circadian rhythm disruption.

12.4.2.3 Memory

As depicted in Fig. 12.3, an intriguing possibility is that circadian rhythm disruption is changing the hippocampal epigenome involved in learning and memory. Histone acetylation, phosphorylation, and DNA methylation occur in the hippocampus after tasks such as contextual fear conditioning and the MWT (Dagnas and Mons 2013; Levenson et al. 2004; Chwang et al. 2006; Rudenko and Tsai 2014; Miller and Sweatt 2007). In accordance with the multiple memory systems account for learning and memory which suggests that there are disparate brain circuits that operate in parallel to mediate different types of memory-based behaviours (McDonald and White 1993; McDonald and Hong 2013), epigenetic modifications induced by learning occur in a region specific manner that depends on the behavioural task (Dagnas and Mons 2013). Remarkably, epigenetics are involved in the interaction between neural circuits, as manipulating histone acetylation in the hippocampus influences whether or not mice use a hippocampal or striatal based strategy in a cue-based navigation task (Dagnas et al. 2013).

Blocking or stimulating these epigenetic modifications impairs, or enhances/rescues hippocampal dependent memory, respectively (Alarcón et al. 2004; Korzus et al. 2004; Rudenko and Tsai 2014; Fischer et al. 2007; Vecsey et al. 2007; Miller and Sweatt 2007; Miller et al. 2010). Epigenetic modifications are also involved in hippocampal dependent memory recall, as amazingly, DNA methylation of genes involved in memory were detected in the anterior cingulate cortex 30 days after acquisition and were necessary for memory recall at this time point (Miller et al. 2010).

As previously mentioned, activation of the signal transduction pathway that facilitates LTP is necessary for learning/memory and epigenetic mechanisms are involved in these pathways. The ability of HDAC inhibitors to enhance (Chwang et al. 2007; Vecsey et al. 2007), or favor a specific neural circuit (Dagnas et al. 2013) are dependent on CREB. Thus, it is not surprising that CREB binding protein is a HAT that is required for hippocampal dependent memory and it appears that this role is dependent on its role as a HAT (Rudenko and Tsai 2014; Alarcón et al. 2004; Korzus et al. 2004). Promoting histone acetylation by inhibiting HDACs can rescue memory by what appears to be late-LTP facilitation (Alarcón et al. 2004).

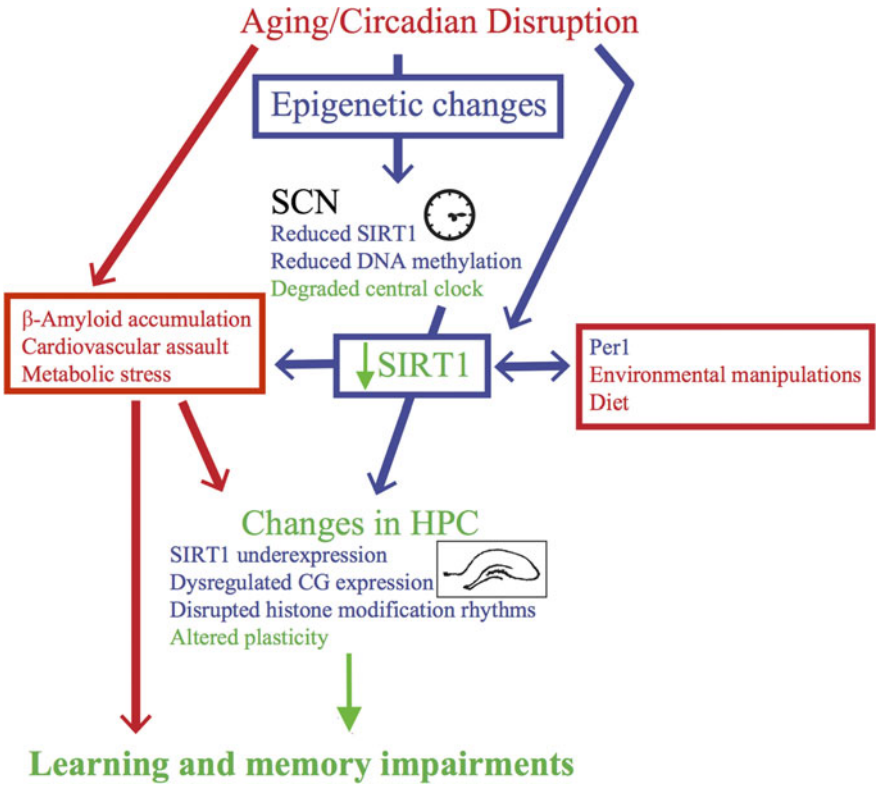

Fig. 12.3 Epigenetic changes are a possible mechanism for the memory impairment induced by circadian rhythm disruption. The blue items are either epigenetic mechanisms or affected by changes in the epigenome, whereas the red items either elicit non-epigenetic mechanisms or are affected by them. The *green items* are believed to be effected by both epigenetic and non-epigenetic mechanisms. Factors that induce circadian rhythm disruption, such as aging or shift work, change the epigenome in the SCN, and these changes contribute to SCN dysfunction. SCN dysfunction then affects the epigenome and/or clock gene (*CG*) expression in peripheral oscillators, such as the hippocampus (*HPC*), which contribute to learning and memory impairments. Other factors, such as diet and environmental manipulations could also create learning and memory impairments by changing the epigenome in the hippocampus. It should be noted however that some of these environmental manipulations might also influence circadian rhythms. Alternatively, in addition to circadian rhythm disruption, the memory impairment induced by aging could be a result of other risk factors that impact hippocampal functioning. However, these risk factors are likely exacerbated by circadian rhythm disruption, and this effect could be mediated by changes in the epigenome (figure and figure caption modified from Deibel et al. 2015 with permission)

Similarly the HDAC, SIRT1, which is involved in the core circadian clock architecture, influences CREB and another molecule critical for learning and memory [brain derived neurotropic factor (BDNF)], via its regulation of miRNA-134 (Gao et al. 2010; Rudenko and Tsai 2014).

Although scarce, several recent papers are suggesting that epigenetics are likely involved in the circadian rhythm induced memory impairment. Recent evidence suggests that the HDAC SIRT1 is likely involved in this story. First, as mentioned above it is involved in both circadian rhythms and memory. Mutant mice with deletions of the HDAC, SIRT1 have impaired circadian rhythms, memory, and LTP (Gao et al. 2010; Michán et al. 2010; Chang and Guarente 2013). The clock is directly involved in SIRT1 function, as learning induced epigenetic changes and hippocampal oscillations of SIRT1 and other histone modifiers are abolished in *Per1* mutant mice (Rawashdeh et al. 2014). While, to our knowledge the effects of environmental circadian rhythm disruption on SIRT1 expression in the hippocampus have not been explicitly investigated, there are several studies which may have addressed this question by indirectly disrupting circadian rhythms. Sleep deprivation, which can alter circadian rhythmicity (Antle and Mistlberger 2000; Deboer et al. 2007), impaired rapid acquisition in the MWT and decreased hippocampal SIRT1 expression in rats (Chang et al. 2009). Similarly, a high fat diet, which can alter circadian rhythms at the molecular and behavioural level (Kohsaka et al. 2007), impaired hippocampal dependent memory that was again thought to be a result of decreased SIRT1 expression in the hippocampus (Heyward et al. 2012). Although these studies did not assess circadian rhythm disruption directly, as clock genes are directly involved in hippocampal SIRT1 expression and these animals were almost certainly experiencing some degree of circadian rhythm disruption, circadian rhythm induced changes in hippocampal SIRT1 expression is a very plausible mechanism that warrants direct investigation.

The epigenomes in the hippocampus and SCN are also affected by aging and one possibility is that the circadian rhythm disruption that occurs during aging is contributing to an altered hippocampal epigenome (Deibel et al. 2015). For example, learning induced epigenetic modifications are abolished in aged mice (Dagnas and Mons 2013; Peleg et al. 2010), as is the ability for HDAC inhibitors to influence which memory structure governs behavioural control (Dagnas et al. 2013). Disrupted circadian rhythms and the inability to entrain to a new photoperiod were associated with decreased expression of a primary DNA methyltransferase (involved in DNA methylation) in the SCN (Azzi et al. 2014).

SIRT1 is crucially involved in aging, an effect that is thought to be partially mediated by the role of its cofactor—NAD^+—in regulating cellular energy (Jenwitheesuk et al. 2014). Aged mice also have decreased SIRT1, BMAL1, and PER2 expression in the SCN, and remarkably, increasing SIRT1 expression in aged mice partially rescues the ability of aged mice to entrain to a new photoperiod (Chang and Guarente 2013). Hippocampal SIRT1 protein levels are also decreased in aged rats due to what appears to be changes in post-translational modifications as SIRT1 mRNA is unaffected (Quintas et al. 2012). In addition to SIRT1 deficient mutant mice having impaired circadian rhythms and memory, they also display premature aging (Wang et al. 2016). Interestingly, these mutant mice and aged mice have increased PER2 expression in the liver, and this increased elevated PER2 expression is directly involved in the aging phenotype displayed by SIRT1 mutant mice (Wang et al. 2016). SIRT1 and PER2 were found to negatively regulate each

other, with age induced reductions in SIRT1 leading to increased expression of PER2 and other molecules that promote aging (Wang et al. 2016).

SIRT1 is a particularly attractive mechanism for the memory impairment induced by circadian rhythm disruption because of its role in calorie restriction. Calorie restriction promotes healthy aging, with aged rodents exposed to calorie restriction having better cognition, longevity, and protection from neurodegenerative diseases (Jenwitheesuk et al. 2014; Moskalev et al. 2014). For example, aged rats that have been exposed to life-long calorie restriction are saved from age-related deficits in hippocampal dependent memory and the plasticity mechanisms thought to underlie it (Pitsikas and Algeri 1992; Eckles-Smith et al. 2000). Interestingly, calorie restriction also affects circadian rhythms at the behavioural and molecular level (Damiola et al. 2000; Hara et al. 2001; Mendoza et al. 2005; Froy and Miskin 2010). It has been suggested that the improved longevity elicited by calorie restriction is partially a result of improved circadian rhythmicity (Froy and Miskin 2007, 2010; Gutman et al. 2011). SIRT1 is a likely involved, as calorie restriction increases protein levels in the hippocampus of aged rats (Quintas et al. 2012), and in the SCN, hippocampus, and cortex of aged mice (Chen et al. 2008; Satoh et al. 2010). It is unknown if calorie restriction or artificial elevation of SIRT1 would have also have beneficial effects on memory that is a result of environmental induced circadian misalignment.

12.4.3 Other Mechanisms

12.4.3.1 Hormones

Stress is influenced by circadian rhythms with the primary stress hormones (glucocorticoids) being expressed in a circadian manner (Zelinski et al. 2014a; Dickmeis 2009; Chrousos 1998). Elevated glucocorticoids can impair hippocampal dependent learning and memory (Shors 2006). Aging is associated with changes in glucocorticoids in humans and animals (Pandi-perumal et al. 2002; Zisapel et al. 2005; Sapolsky and Altmann 1991). Our group demonstrated that hamsters with age-induced dampening of baseline and corticosterone rhythms had poorer hippocampal dependent memory than age-matched controls with better corticosterone rhythms Cain et al. (2004b). Although correlative, these data open the possibility that the memory impairment induced by circadian rhythm disruption involves an altered stress response (Zelinski et al. 2014a; Deibel et al. 2014a).

In rodent models that utilize shifts of the light dark cycle to disrupt circadian rhythms, base-line corticosterone which is the equivalent to cortisol in humans, is typically not elevated after circadian rhythm disruption (Castanon-Cervantes et al. 2010; Loh et al. 2010; Logan et al. 2012; Sei et al. 2003). However, corticosterone was elevated in hamsters when samples were taken during circadian rhythm disruption (Gibson et al. 2010). Similarly, in our paradigm, corticosterone was increased towards the end of our chronic paradigm, but not after acute or

intermediate amounts of phase advances (Deibel et al. 2014b). This suggests that elevated corticosterone is likely not the primary mechanism for our observed impairment because it was not elevated during the acute shift. However, we only assessed baseline corticosterone during the nadir of the rhythm. Therefore as documented in similar mouse paradigms, we cannot rule out the possibility that the corticosterone rhythm was out of phase with the light dark cycle (Barclay et al. 2012), nor that the stress response was exacerbated when exposed to a stressful event (Loh et al. 2010). These possibilities need to be examined in our paradigm to fully elucidate the contribution of stress to our observed memory impairment.

Melatonin, aptly named the "hormone of darkness", is secreted from the pineal gland and is an example of another hormone that is secreted in a circadian manner (Jenwitheesuk et al. 2014; Rawashdeh and Maronde 2012). Although melatonin is an output of the circadian system it is able to affect the functioning of both the master and peripheral clocks as there are melatonin receptors in the SCN and brain areas involved in learning and memory such as the hippocampus, subiculum, and entorhinal cortex (Jenwitheesuk et al. 2014; Rawashdeh and Maronde 2012; Krishnan and Lyons 2015; Chaudhury et al. 2005). Melatonin secretion is suppressed by light and thus peak melatonin expression occurs during the nighttime regardless of whether the animal is nocturnal or diurnal (Jenwitheesuk et al. 2014; Rawashdeh and Maronde 2012). As with corticosteroids, rhythmic and basal melatonin expression is blunted during aging (Reiter et al. 1981; Poeggeler 2005; Skene et al. 1990).

As with aging, circadian rhythm disruption dramatically attenuates the melatonin rhythm (Korkmaz et al. 2009; Schwimmer et al. 2014). Melatonin could be a mechanism for the circadian rhythm disruption induced memory impairment (Jenwitheesuk et al. 2014; Deibel et al. 2015). For example, melatonin inhibits LTP by down regulating adenylyl cyclase and subsequently protein kinase A via its action on melatonin type two receptors (MT2) (Wang et al. 2005). Transgenic mice without MT2 receptors have impaired LTP and hippocampal dependent memory (Larson et al. 2006). It has been theorized that melatonin might be involved in some sort of synaptic scaling that involves the suppression of LTP and memory during the nighttime (Wang et al. 2005). Findings that LTP is still rhythmic in mice lacking it, and the fact that memory and LTP can be stronger in rodents at night despite melatonin being at its peak, suggest that this is a complex story (Chaudhury et al. 2005). An intriguing possibility is that during circadian misalignment, learning and memory could be affected by a mistimed melatonin rhythm. Further studies involving circadian rhythm misalignment are required to determine exactly how melatonin contributes to learning and memory.

Melatonin is also thought to have an epigenetic effect, particularly in the silencing of gene expression (Korkmaz et al. 2009; Jenwitheesuk et al. 2014). As previously mentioned, exogenous melatonin decreased breast cancer tumor size by rescuing DNA hypomethylation (Schwimmer et al. 2014). Jenwitheesuk and colleagues (Jenwitheesuk et al. 2014) theorized that attenuated melatonin expression due to aging is contributing to the age-associated cognitive impairment via its effect on hippocampal SIRT1 expression. The role of melatonin in both memory and

circadian rhythm regulation suggests that it is a possible target for treatments. The finding that exogenous melatonin partially rescued SCN clock gene expression in aged rats (Mattam and Jagota 2014), and hippocampal SIRT1 expression in sleep-deprived rats (Chang et al. 2009) is particularly encouraging.

12.4.3.2 Sleep

Sleep is another very appealing candidate when considering possible mechanisms for the memory impairment induced by circadian rhythm disruption. A popular theory in the learning and memory field is that sleep is involved in memory consolidation (Diekelmann and Born 2010). Various types of memory in both humans and rats benefit from sleep after learning (Diekelmann and Born 2010). Hippocampal dependent memory consolidation appears to involve non-rapid-eye-movement sleep as blocking aspects of it impair memory (Ego-Stengel and Wilson 2010). In humans, aging is associated with decreased non-rapid-eye-movement sleep, and reductions in two sleep components specifically thought to be involved in memory consolidation: k-complexes and sleep spindles, (Diekelmann and Born 2010; Crowley et al. 2002). With this in mind, one possibility for the memory impairment induced by circadian rhythm disruption is that sleep is being affected in some way (Zelinski et al. 2014a; Deibel et al. 2015). This could be evidenced by changes in the sleep rhythms and/or changes in the amount of sleep.

Similar rodent circadian rhythm disruption paradigms to ours, typically do not find changes in the amount of sleep or it's associated components (Castanon-Cervantes et al. 2010; Sei et al. 1992; Loh et al. 2010), however, sleep can become desynchronized form the light-dark cycle (Altimus et al. 2008; Castanon-Cervantes et al. 2010; Loh et al. 2010; 2015; Sei et al. 1992; Van Dycke et al. 2015). We recently demonstrated that during our acute paradigm, the amount of sleep and it's stages/components does not change and these process are still oscillating in a circadian manner (Rota et al. 2016). However, sleep was rapidly desynchronized from the light dark cycle during the six days of phase advances as was evidenced by less sleep and it's stages/components during the typical inactive phase of the light dark cycle (light phase in rodents) (Rota et al. 2016). It appeared that the rhythms of sleep and its associated components free-ran during the six days of phase advances without showing any evidence of entrainment to the novel light-dark cycle. This circadian misalignment is similar to that which is thought to occur during shiftwork or jetlag and as discussed above has many deleterious effects on the brain and body (Escobar et al. 2011; Zelinski et al. 2014a).

In terms of memory consolidation, it is possible that sleep that is out of phase with the light dark cycle could change the duration between training and sleeping bouts (Rota et al. 2016; Diekelmann and Born 2010). This could affect memory consolidation as some data suggest that the beneficial effects of sleep on memory consolidation are enhanced when sleep occurs shortly after memory acquisition (Diekelmann and Born 2010). For the first time, we also demonstrated that

K-complexes and sleep spindles, which are thought to be involved in the cross-talk between the hippocampus and cortex that might occur during memory consolidation were also out of phase with the light dark cycle during the phase advances (Rota et al. 2016). While uninvestigated in that study, it remains to be seen if desynchrony of sleep spindles and k-complexes from the light dark cycle specifically affects the hippocampus-cortex interaction.

12.4.3.3 Neurogenesis

For an in depth discussion of neurogenesis and circadian rhythms readers are encouraged to see the relevant sections in the review by Smarr and colleagues (Smarr et al. 2014). Neurogenesis is the birth of new neurons in adults, and one of the very few brain areas in which it occurs is the dentate gyrus region of the hippocampus (Smarr et al. 2014). Hippocampal neurogenesis is involved in learning and memory as it is necessary for and increased after hippocampal dependent learning (Epp et al. 2013; Smarr et al. 2014; Snyder et al. 2005). In rats, aging is associated with attenuated neurogenesis and changes in neurogenesis along the septo-temporal axis of the dentate gyrus (Snyder et al. 2011; McDonald and Wojtowicz 2005). Accordingly, there are also circadian rhythms associated with the hippocampal proliferation of new cells, however this can depend on certain variables such as timing of wheel activity and markers used to detect proliferation (Mueller et al. 2011; Smarr et al. 2014). Manipulations that induce circadian rhythm disruption such as clock gene deletions in mutant mice (Borgs et al. 2009), constant light (Fujioka et al. 2011), but see Mueller et al. (2011), or phase shifts can affect neurogenesis in rodents (Gibson et al. 2010; Kott et al. 2012). Speaking to the severity of circadian misalignment, rhythms that are out of phase with the light dark cycle might be more deleterious for hippocampal neurogenesis (Gibson et al. 2010; Kott et al. 2012) than arrhythmicity that is induced by constant illumination (Mueller et al. 2011).

An elegant set of experiments utilizing body, nervous system specific, and inducible stem cell specific deletions of SIRT1, determined that SIRT1 is involved in regulating hippocampal neurogenesis via it's inhibitory effect on cell proliferation and self-renewal of adult neural stem cells (Ma et al. 2014). It is possible that the changes in neurogenesis elicited by circadian rhythm disruption are mediated by changes in SIRT1 expression in the hippocampus. Further studies are encouraged to investigate SIRT1 in situations of circadian arrhythmicity, misalignment, and aging-related circadian rhythm disruption.

12.4.3.4 Cofactor Theory

Our lab theorizes that the cognitive impairments that occur in aging and sporadic AD (no genetic precursors) are a result of the interaction of various risk factors that affect areas of the brain involved in learning and memory (McDonald 2002; Craig

et al. 2011; Gidyk et al. 2015; McDonald et al. 2010). These factors can interact synergistically, with some factors making the brain more susceptible to other factors (Craig et al. 2011; McDonald 2002; Gidyk et al. 2015; McDonald et al. 2010). In rodent models this can be evidenced by brain pathology and cognitive impairments only when certain risk factors are presented in tandem and not in isolation (Craig et al. 2011; Gidyk et al. 2015; McDonald 2002; McDonald et al. 2010). Circadian rhythm disruption could make the hippocampus more susceptible to damaging agents (Craig et al. 2011; McDonald 2002; Gidyk et al. 2015; McDonald et al. 2010). Preliminary evidence from our lab supports this notion, as hippocampal pathology was exacerbated by a small stroke when given to rats that experienced our acute circadian rhythm disruption paradigm (Gidyk et al. 2015). But as with other risk factors used in our lab, these effects vary depending on the specific factors used as circadian rhythm disruption did not exacerbate the effects of cholinergic depletion of the medial septum (Gidyk et al. 2015; Craig et al. 2009).

SIRT1 could be involved in the effect that various risk factors have on the hippocampus. It is a neuroprotective agent that fights various brain pathologies in AD, such as amyloid beta formation (Jenwitheesuk et al. 2014; Brunet and Berger 2014; Kim et al. 2007; Qin et al. 2008). With this in mind, we have demonstrated that the effects of a stroke on the hippocampus are exacerbated in aged rats (Driscoll et al. 2008). Although untested, it remains a possibility that in our models, aging and circadian rhythm disruption are reducing hippocampal SIRT1 expression, which could make the brain more susceptible to damaging agents.

12.5 Conclusions

In summary, epigenetics provide a way for the environment to influence the brain and subsequently behaviour. As circadian rhythms and memory are plastic processes that constantly require the integration of information from the environment, it is not surprising that epigenetics is integral to both of these processes. Chronic circadian rhythm disruption occurs in various occupations, but is also involved in natural aging, and age-associated diseases such as AD. Preliminary evidence suggests that circadian rhythms disruption can change the epigenome in the SCN and hippocampus. Epigenetic mechanisms are likely interacting with other mechanisms involved in the circadian rhythm disruption induced memory impairment. Epigenetics is a particularly appealing mechanism due to its preliminary efficacy as a target for treatments. Circadian rhythms and memory are improved by both exogenous melatonin, and calorie restriction, which partially appears to be due to restoring epigenetic function in the SCN and hippocampus. Much is yet to be determined regarding the exact role of epigenetics in circadian rhythm induced memory impairments and it is our opinion that experiments that induce circadian misalignment will be crucial to defining this role.

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Chapter 13
Circadian Sleep-Wake Activity Patterns During Aging

Katie L. Stone and Gregory J. Tranah

Abstract Aging is associated with altered circadian activity rhythms, the intrinsic physiologic cycles of approximately 24 h that are critically involved in control of sleep-wake cycles and numerous physiological processes. Measurement of 24-h patterns of activity is relatively simple and cost-effective using actigraphy. There is growing evidence that older adults with disrupted 24-h activity patterns (weak, more fragmented, and shifted circadian activity rhythms) have higher risk for a variety of age-related outcomes including earlier mortality, risk of cognitive impairment or risk of developing mild cognitive impairment and dementia, cardiovascular disease, depression and anxiety, and mortality risk. Further study is needed to establish mechanisms for these associations. In addition, future studies will be needed to test whether interventions (e.g. physical activity, bright light exposure, cataract surgery) that regulate circadian activity rhythms will improve health outcomes in the elderly.

Keywords Circadian activity rhythms · Aging · Epidemiology · Actigraphy

13.1 Circadian Activity Rhythms and Aging

Circadian rhythms are intrinsic physiologic cycles of approximately 24 h that are critically involved in control of sleep-wake cycles and numerous physiological processes. Several biological functions are under circadian control, including release of certain hormones, body temperature, blood pressure and heart rate, bone remodeling, and sleep and activity cycles (rest activity rhythm). The sleep-wake cycle is synchronized to the time of day by a number of cues, the strongest of which is the environmental rhythm of light and darkness. Aging is associated with altered

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circadian activity rhythms including decreased amplitude (height of rhythm) (Kripke et al. 2005), fragmentation or loss of rhythms (weakening of rhythmic pattern) (Buysse et al. 2005; Czeisler et al. 1992; Duffy et al. 2002; Weitzman et al. 1982; Yoon et al. 2003; Sakurai and Sasaki 1998; Luik et al. 2013), shortened natural free-running period (period of rhythm without environmental cues), a tendency towards internal desynchronization (Carrier et al. 1996; Hofman and Swaab 2006; Munch et al. 2005), and decreased sensitivity to phase resetting signals, including light and sleep medications (Hofman and Swaab 2006). The timing of peak rhythm activity also advances with age resulting in an earlier onset of sleepiness in the evening, and an earlier morning waking time (Czeisler et al. 1992). The disruption of circadian rhythms during aging is paralleled by decreased photic input both because of age-related losses in circadian photoreception (Turner and Mainster 2008) and because older people tend to be exposed to less light (Campbell et al. 1988). It has been hypothesized that disruptions in these rhythms may occur in part, as a result of age-related deterioration of the SCN (Saper et al. 2005). Animal studies suggest that aging is also associated with a number of molecular changes in the circadian system including changes in expression patterns of clock genes and changes in the neurochemistry of the SCN (Hofman and Swaab 2006; Wyse and Coogan 2010; Hofman and Swaab 1994; Kolker et al. 2003).

Little is known concerning the causes of age-related changes in circadian patterns and the subsequent effects of these changes on health and well-being. Circadian and sleep-wake rhythm abnormalities have been observed among those with a wide variety of medical conditions. Activity phase abnormalities in the elderly with dementia have been shown to predict a shorter survival (Gehrman et al. 2004). Disturbances of the sleep-wake cycle, which are reflected in poor activity rhythms, are particularly pronounced in Alzheimer's disease (AD) (Satlin et al. 1995; Gehrman et al. 2005; Ancoli-Israel et al. 1997) and hypothesized to be one of the primary causes of institutionalization (Bliwise 1993; Van Someren 2000) along with shifts in daytime patterns (Pollak and Perlick 1991). Patients diagnosed with AD also exhibit circadian disturbances, including reduced amplitudes and phase delay of circadian variation in core body temperature and activity (van Someren et al. 1996). Survival in patients with metastatic colorectal cancer was 5 times higher among those with stronger circadian activity rhythms than in those with rhythm abnormalities (Mormont et al. 2000). Furthermore, those with more daytime compared to nighttime activity had better quality of life (Mormont et al. 2000; Mormont and Waterhouse 2002). Activity phase abnormalities in the elderly with dementia have been shown to predict shorter survival (Gehrman et al. 2004). However, it is not clear whether activity rhythms are directly influencing morbidity and mortality or represent biomarkers of advanced physiological aging that provide additional risk beyond that of traditional covariates. Indeed, van Hilten et al. (1993) examined the influence of age on nocturnal behavior in 100 healthy older adults and concluded that without illness, age itself has only marginal effects on sleep and wake. In this chapter we review the latest evidence from large, population-based cohorts of elderly participants that alterations in circadian activity rhythms are associated with mortality (Tranah et al. 2010; Paudel et al. 2010), dementia and

mild cognitive impairment (Tranah et al. 2011), cardiovascular disease (Paudel et al. 2011), and depression (Luik et al. 2013; Maglione et al. 2014).

13.2 Measurement of Sleep-Wake Activity Rhythms

There are a variety of methodologies for analyzing circadian activity rhythms, (Luik et al. 2013; Tranah et al. 2010; Paudel et al. 2010; Middleton et al. 1997; Martin et al. 2000; Ancoli-Israel et al. 2002, 2003a; Van Someren et al. 1999; Marler et al. 2006) but no single standard methodology has been accepted. Actigraphy records limb movements and allows for 24-h recordings of activity from which wake and sleep can be scored. Traditionally, the actigraphs are placed on a wrist, although sometimes activity from the leg or waist is recorded. Once actigraphic data are collected, the signal is displayed on a computer and examined for patterns of activity/inactivity and analyzed for wake/sleep. Wrist activity has the advantages of being cost-efficient, allowing the recording of sleep in natural environments, recording behavior that occurs both during the night and during the day, and recording for long time periods. While not a replacement for electroencephalogram (EEG) or polysomnography (PSG) recording of sleep behavior or circadian measures based on 24-h melatonin or cortisol, there are times when actigraphy provides clear advantages for data collection. Activity is a valid marker of entrained PSG sleep phase and correlates strongly with entrained endogenous circadian phase (Ancoli-Israel et al. 2003b). It is also useful in identifying sleep that has been disturbed by circadian rhythm changes. Actigraphy allows the study of rhythms occurring over many days; it is therefore well suited for the study of circadian rest activity rhythms. Actigraphy is also a useful adjunct to the routine evaluation of circadian rhythm disorders. Another advantage is that actigraphy can be deployed in large clinical studies to examine the role of activity rhythms in health and morbidity as well as for tracking interventions targeting rhythm disturbances.

The wrist actigraph may be particularly valuable for studying individuals who have difficulty sleeping in a sleep laboratory or sleeping with the wires associated with traditional PSG, such as insomniacs, children and demented elderly. With actigraphy, patients are studied in their natural environment. Mormont et al. (2000) used actigraphy to study circadian rhythms and sleep/wake cycles in cancer patients, as a preliminary step towards the advancement of chronotherapy. Studies have also been done examining sleep schedules of adolescents (Usher et al. 1999), shift workers (Walsh et al. 2014; Luik et al. 2015a), in-flight crews (Scheer et al. 2009), and jet lag (Zuurbier et al. 2015).

Three large, population-based studies of elderly individuals have measured activity rhythms using wrist actigraphy: Study of Osteoporotic Fractures (SOF), Osteoporotic Fractures in Men (MrOS), and the Rotterdam Study (RS). The SOF and MrOS studies collected activity data with the Sleep-watch-O (SleepWatch-O®, Ambulatory Monitoring, Inc) actigraph and studied activity rhythm association with mortality (Tranah et al. 2010; Paudel et al. 2010), dementia and mild cognitive

impairment (Tranah et al. 2011), cardiovascular disease (Paudel et al. 2011), and depression (Maglione et al. 2014). In these two studies, an extension to the traditional cosine model was used to map the circadian activity rhythm to the activity data (Marler et al. 2005). The following activity rhythm parameters were calculated from the extended cosine curve: Amplitude, an indicator of the strength of the rhythm, is the peak to nadir difference in activity (measured in arbitrary units of activity [counts/min]); Mesor, mean level of activity (measured in arbitrary units of activity [counts/min]); Pseudo F-value, a measure of model fit, with smaller values indicating a less robust rhythmic pattern in rest/activity and hence overall reduced circadian rhythmicity; Acrophase, timing of peak activity measured as time of day; Beta (β) statistic, a measure of steepness of the curve, in which larger values represent more square-shaped waves, which would suggest a more constant level of daytime activity; Alpha (α) statistic, a measure of peak-to-trough width, in which small values would represent curves where the troughs are narrower than the peaks, suggesting greater a daytime to nighttime activity ratio; and Minimum, the lowest modeled activity in which large values may be indicative of greater night-time activity. The RS collected activity data with the Actiwatch model AW4 (Cambridge Technology, Cambridge UK) and examined several correlates of activity rhythms including depression (Luik et al. 2013). The following activity rhythm parameters were calculated in the RS: M10 onset, onset of 10 most active hours (proxy of acrophase); L5 onset, onset of 5 least active hours; Amplitude, difference between average activity level in M10 and L5 periods; Relative amplitude, amplitude divided by sum of M10 and L5; Average, average of activity over the day (proxy of mesor); Interdaily stability, degree of regularity in rhythm over days; and Interadaily variability, fragmentation of the rhythm.

13.3 Circadian Rest-Activity Rhythms and Age-Related Outcomes

13.3.1 Mortality

A disrupted or less robust circadian activity rhythm has been associated with medical illness, such as dementia and cancer. Indeed, disturbances of the sleep/wake cycle, which are reflected in poor activity rhythms, are hypothesized to be one of the primary causes of institutionalization (Bliwise 1993; Van Someren 2000). While the association between circadian activity rhythms and illness is fairly strong, evidence for an association between disrupted activity rhythms and subsequent mortality is limited (Gehrman et al. 2004; Mormont et al. 2000; Mormont and Waterhouse 2002). Tranah et al. (2010) examined whether circadian activity rhythms are associated with mortality in 3027 community-dwelling women from the SOF cohort (mean age 84 ± 4 years, range 77–99 years). Activity data were collected for a minimum of three 24-h periods and vital status, with cause of death

adjudicated through death certificates, was prospectively ascertained. Over an average of 4.1 years of follow-up there were 444 (15%) deaths. Those women with lower peak (amplitude) and mean (mesor) levels of activity and less robust rhythms had the shortest overall survival and the risks of all-cause mortality increased from highest to lowest quartile of amplitude. Compared to the highest quartiles, an approximately 2-fold adjusted higher risk of all-cause mortality was observed for those in the lowest quartiles of amplitude (Hazard ratio [HR] = 2.18, 95% CI, 1.63–2.92), mesor (HR = 1.71, 95% CI, 1.29–2.27), and rhythm robustness (HR = 1.97, 95% CI, 1.50–2.60) after multivariate adjustment for age, clinic site, race, body mass index (BMI), cognitive function, sleep performance, exercise, instrumental activities of daily living (IADL) impairments, depression, medications, alcohol, smoking, self-reported health status, married status, and co-morbidities. An increased risk of atherosclerotic mortality was also observed for the lowest quartiles of amplitude (HR = 1.81, 95% CI, 1.13–2.90) and mesor (HR = 1.61, 95% CI, 1.02–2.54) activity levels as well as the lowest quartile of rhythm robustness (HR = 2.31, 95% CI, 1.45–3.68). The relationships with atherosclerotic mortality were largely driven by associations between circadian activity rhythms and either coronary heart disease (CHD) or stroke mortality, which comprised 34 and 32% of atherosclerotic mortality cases, respectively. Increased risk of "other-cause" mortality was observed in the lowest quartiles of amplitude (HR = 3.11, 95% CI, 1.93–5.00), mesor (HR = 2.09, 95% CI, 1.33–3.28), and rhythm robustness (HR = 2.17, 95% CI, 1.43–3.31), compared with the highest quartiles. Further analysis excluding the two most common causes of "other cause" mortality (pulmonary and cognitive causes) produced similar results, suggesting that these two causes of death do not explain this association.

Acrophase was examined in terms of the deviation from the population mean. We identified three categories based on having a peak time of more than 1.5 standard deviations (SDs) above and below the population mean for the study population. Phase advanced participants were defined as having an acrophase of <12:50 PM (−1.5 SD from the mean) and phase delayed participants were defined as having an acrophase of >4:33 PM (+1.5 SD from the mean). The mean peak range was 12:50 PM–4:33 PM. Acrophase deviation was not associated with all-cause or "other cause" mortality. A delayed acrophase was associated with increased cancer (HR = 2.09, 95% CI, 1.04–4.22) and stroke (HR = 2.64, 95% CI, 1.11–6.30) mortality when compared to the mean peak range.

Paudel et al. (2010) examined whether circadian activity rhythms are associated with mortality in 2964 community-dwelling men from the MrOS cohort (mean age 76 ± 6 years). Activity data were collected for a minimum of five 24-h periods and vital status, with cause of death confirmed with death certificates and additionally with medical records when available, was prospectively ascertained. Over an average of 3.5 years of follow-up there were 233 (8%) deaths. After adjustment for multiple potential confounders (age, age^2, race, alcohol use, smoking status, caffeine use, education, self-reported health status, IADL impairments, cognitive impairment, depression, and number of medical conditions), men in the lowest quintile of rhythm robustness had a 57% higher mortality rate (HR = 1.57, 95% CI,

1.03–2.39) compared with men in the highest quintile. This association among those in the lowest quintile of rhythm robustness was even stronger with increased risk of cardiovascular disease (CVD)-related mortality (HR = 2.32, 95% CI, 1.04–5.22). Additionally, men in the latest quintile of acrophase (>3:09 PM) had a 2.8-fold higher rate of CVD-related mortality (HR = 2.84, 95% CI, 1.29–6.24) when compared to those with mean acrophase timing (1:58 PM–2:32 PM). In general, amplitude, mesor and rhythm robustness steadily declined with advancing age. However, there was no association between amplitude and mesor and risk of CVD-related, cancer-related or other causes of mortality.

Further evidence of an association between circadian activity rhythms and mortality risk were reported in the Rotterdam cohort (Zuurbier et al. 2015). Among 1734 men and women aged 45–98, wrist actigraphy and sleep diaries were used to assess objective and subjective measures of sleep, including traditional sleep-wake exposures as well as 24-h parameters including intraday stability (how similar the day/night patterns are over several days) and intraday variability (rate of shifting between rest and activity). A total of 154 deaths (8.9%) were observed over approximately 7 years of follow-up. Based on Cox proportional hazards analysis (adjusting for age, sex, BMI, smoking, comorbidities, medication use, cognitive function, depression, apnea and napping status) individuals with higher levels of intraday stability had lower risk of mortality (HR = 0.83; 95% CI, 071–0.96 per SD). In contrast, those with more fragmented rhythms (more intraday variability) had greater mortality risk (HR = 1.22; 95% CI 1.04–1.44 per SD). Results were similar in sensitivity analyses excluding deaths that occurred in the first year after sleep exposure assessment. Traditional sleep-wake parameters, including sleep duration, wake after sleep onset, and sleep onset latency were not associated with mortality after multivariate adjustment. The timing of activity rhythms was not examined.

13.3.2 Dementia and Mild Cognitive Impairment

Circadian rhythms play an important role in rhythms associated with cognitive processing including alertness, learning and memory. During waking, there are observed rhythms in synaptic plasticity (Chaudhury et al. 2005; Chaudhury and Colwell 2002), focused attention and behavioral flexibility (Aston-Jones and Cohen 2005; Usher et al. 1999). Little is known concerning the causes of age-related changes in circadian activity patterns in non-demented older adults and the subsequent effects of these changes on health and well-being. Tranah et al. (2011) examined whether circadian activity rhythms are associated with incident mild cognitive impairment (MCI) and dementia 1282 women from the SOF cohort with both actigraphy data and a follow-up visit that included an expanded cognitive assessment and adjudication of MCI-dementia by an expert panel. After a mean of 4.9 years of follow-up from the collection of actigraphy data, there were 195 (15%) women with dementia and 302 (24%) with MCI ascertained by adjudication. An

approximately 50% adjusted higher odds of developing dementia or MCI versus those without any dementia or MCI was observed for those in the lowest quartiles (n = 320) of amplitude (Odds ratio [OR] = 1.54, 95% CI, 1.07–2.21) and rhythm robustness (OR = 1.55, 95% CI, 1.08–2.22) when compared to those highest quartiles (n = 322).

Acrophase was examined in terms of the deviation from the population mean. Three categories were defined based on a peak time of more than 1 standard deviation (SD) above and below the population mean for the study population. Phase advanced participants were defined as having an acrophase of <1:34 PM (−1 SD from the mean) and phase delayed participants were defined as having an acrophase of >3:51 PM (+1 SD from the mean). Acrophase deviation was associated with increased odds of developing dementia or MCI. A delayed acrophase, after 3:51 PM (n = 173, 13%), was associated with a significant increase in odds of developing dementia or MCI (OR = 1.83, 95% CI, 1.29–2.61) when compared to the mean peak range of 1:34 PM–3:51 PM (n = 927, 73%). An advanced acrophase, before 1:34 PM (n = 182, 14%), was associated with an elevated but non-significant odds of developing dementia or MCI (OR = 1.36, 95% CI, 0.96–1.92) when compared to the mean peak range. All analyses were multivariate adjusted for age, clinic site, race, education, BMI, walking for exercise, functional status, depression, benzodiazepine and antidepressant use, sleep medication use, alcohol use, caffeine intake, smoking, self-reported health status, and prior medical conditions. Results for all models were unchanged after further adjustment for sleep efficiency.

In an extension of this study, Walsh et al. (2014) examined whether circadian activity rhythms are associated with executive function 1287 women from the SOF cohort. Baseline actigraphy was used to determine circadian activity rhythm measures and cognitive performance was assessed five years later and included: Modified Mini-Mental Status Examination (3MS), California Verbal Learning Task (CVLT), digit span, Trail Making Test B (Trails B), categorical fluency, and letter fluency. Women in the lowest quartile for amplitude performed worse on Trails B and categorical fluency compared to women in the highest quartile (group difference (d) = 30.42 s, d = −1.01 words respectively, $P < 0.05$). Women in the lowest quartile for mesor performed worse on categorical fluency (d = −0.86 words, $P < 0.05$). Women with a later acrophase performed worse on categorical fluency (d = −0.69 words, $P < 0.05$). Controlling for baseline 3MS and sleep factors had little effect on our results.

Additional evidence linking activity rhythms and cognitive function is reported in a cross-sectional analysis of 24-h activity rhythms and both global as well as specific domains of cognitive function. For this analysis, Luik and colleagues utilized data from 1723 middle-aged and older adults (mean age 62 ± 9.4 years) from the Rotterdam study cohort (Luik et al. 2015a). Using activity patterns based on wrist-worn actigraphs, both 24-h rhythms (stability and fragmentation of rhythms) as well as traditional sleep parameters were examined. Cognitive performance was assessed using the Letter Digit Substitution Test (LDST: processing speed), Stroop Test (executive function), Word Learning Test (memory) and Word Fluency Test

(verbal fluency). Global cognition was also examined as a composite of the individual cognitive tasks. The results showed that 24-h rhythms were significantly related to performance on executive function and speed. In particular, after multivariate adjustment (age, sex, employment status, education, BMI, and other health and comorbidities), those with less stable rhythms had significantly worse scores on both the LDST and the Stroop Test (p-values = 0.004 and 0.003, respectively). Similarly, those with more fragmented rhythms experienced worse performance on the LDST and Stroop. Furthermore, significant effect modification by age was observed, such that both sleep stability and sleep fragmentation were more strongly associated with cognitive performance among older adults, and less strongly associated among younger (middle-aged) adults. In contrast, traditional sleep variables (in particular, sleep onset latency) were significantly related to performance on tests of memory and verbal fluency. In general, both 24-h activity rhythms and sleep exposures were associated with global cognition.

13.3.3 Cardiovascular Disease

Previous studies are consistent in suggesting that disruptions in circadian activity rhythms are associated with adverse cardiovascular consequences, such as CVD, atherosclerotic, and stroke-related mortality (Tranah et al. 2010, 2011), as well as short-term consequences in metabolic measures (Scheer et al. 2009). While disrupted circadian activity rhythms and CVD events are both more common in the elderly, little is known regarding associations between circadian activity rhythm variables and risk of CVD-related events in older adults. Paudel et al. (2011) examined whether circadian activity rhythms are associated with risk of cardiovascular disease CVD events in 2968 men from the MrOS cohort. Over an average of 4.0 years of follow-up there were 490 (16.5%) CVD events. Increased risk of CVD events was observed among participants with reduced amplitude (HR = 1.31, 95% CI, 1.01–1.71) and greater minimum (HR = 1.33, 95% CI, 1.01–1.73). In addition, reduced amplitude (HR = 1.36, 95% CI, 1.00–1.86) and greater minimum activity counts (HR = 1.39, 95% CI, 1.02–1.91) were associated with increased risk of CHD events. Reduced rhythm robustness (HR = 2.88, 95% CI = 1.41–5.87) was also associated with an increased risk of peripheral vascular disease events when the lowest quartile was compared with the highest quartile. Men in the lowest quartile of β (indicating less constant levels of daytime activity) had a 78% increased risk of incident stroke compared with men in the highest quartile in multivariable adjusted models (HR = 1.78, 95% CI = 1.01–3.14). Additional analyses excluding men who reported having had prevalent CVD disease or models adjusting for traditional CVD risk factors such as diabetes, blood pressure, total cholesterol, and HDL did not affect the statistical significance of results.

13.3.4 Depression

There is considerable evidence to support a role for circadian rhythm disturbances in the pathophysiology of depression (Germain and Kupfer 2008; McClung 2011; Monteleone et al. 2011). Circadian rhythm disturbances have been observed in unipolar depression, bipolar depression, and seasonal affective disorder (Kasper and Hamon 2009; Lamont et al. 2007). In unipolar depression multiple rhythms are disrupted including prolactin, cortisol, growth hormone, melatonin, body temperature, and the sleep/wake cycle (Linkowski et al. 1985). At the neuroanatomical level, depressed patients have been shown to have different patterns of regional brain glucose metabolism throughout the day compared with normal patients and the expression of enzymes that control catecholamine metabolism may be regulated by clock genes (Hampp and Albrecht 2008; Hampp et al. 2008). Although aging promotes disruption in circadian rhythms, the relationship between depression and circadian rhythm disruption in older adults remains largely unexplored. Maglione et al. (2014) examined the relationship between depressive symptoms assessed with the Geriatric Depression Scale (GDS) and circadian activity rhythms in older adults among 3020 women (mean age 83.6 ± 3.8 years) from the SOF cohort. Greater levels of depressive symptoms on the GDS were associated with greater desynchronization (decreased amplitude, rhythm robustness, and mesor) of circadian activity rhythms in linear regression models. In addition, greater levels of depressive symptoms were associated with a later average time becoming active in the morning but not with acrophase. When categorizing participants by GDS score as "normal" (0–2; referent group, n = 1961), "some depressive symptoms" (3–5, n = 704), or "depressed" ($\geq$6, n = 355), Maglione et al. (2014) assessed the odds falling into the lowest quartile for activity rhythm variables. Risk of falling into the lowest quartile of amplitude was elevated for both those defined "some depressive symptoms" (OR = 1.32, 95% CI, 1.04–1.67) or "depressed" (OR = 1.51, 95% CI, 1.11–2.05). In addition, risk of falling into the lowest quartile of mesor was elevated for both those defined "some depressive symptoms" (OR = 1.55, 95% CI, 1.24–1.94) or "depressed" (OR = 1.62, 95% CI, 1.20–2.18). Additional adjustment for total sleep time and sleep efficiency did not affect the statistical significance of results. In an extension of this work, Maglione et al. (2015) examined the relationship between circadian gene polymorphisms and depressive symptoms in older adults from the SOF and MrOS studies. In a cross-sectional genetic association study of 529 single nucleotide polymorphisms (SNPs) representing 30 candidate circadian genes, Maglione et al. identified PER3 SNPs associated with decreased odds of reporting "some depressive symptoms" and a single RORA SNP associated with greater odds of reporting "many depressive symptoms". These associations were identified through meta-analysis of both studies and met multiple testing criteria for statistical significance. Luik et al. (2013) examined activity rhythm stability and fragmentation in 1734 middle-aged and elderly participants (mean age 62 ± 9.4 years) from the Rotterdam Study cohort. Activity data were collected for a minimum of four 24-h periods and investigated age, sociodemographics, mental

health, lifestyle (coffee use, alcohol use, and smoking), and sleep characteristics as determinants of activity rhythms of activity. The results of this study indicate that older age is associated with a more stable 24-h activity rhythm, but also with a more fragmented distribution of periods of activity and inactivity. Both BMI and smoking were associated with less stable and more fragmented activity rhythms. In concordance with the results from the SOF cohort (Maglione et al. 2014), more depressive symptoms were related to less stable and more fragmented activity rhythms. In a separate analysis based on the same dataset (Luik et al. 2015b), Luik and colleagues reported that more fragmented activity rhythms were associated cross-sectionally with clinically significant depression and anxiety. For each standard deviation increase in 24-h fragmentation, there was a 1.27-fold increase in odds of depression (Odds Ratio [OR] = 1.27; 95% CI 1.04–1.54), and a 1.39-fold increase in anxiety (OR = 1.39; 95% CI = 1.14–1.70). In contrast, there were no significant associations between rhythm stability or timing, or traditional sleep-wake exposures and risk of depression or anxiety after full adjustment.

Further evidence of an association between circadian activity rhythms and risk of depression was reported in a longitudinal analysis by Smagula et al. (2015). In this longitudinal study of community-dwelling older men, activity rhythms from wrist actigraphs were analyzed using a 5-parameter modified cosine model, and depressive symptoms were assessed at baseline and 5 years later using the Geriatric Depression Scale (GDS), which ranges from 0 to 16 with higher scores indicating more depressive symptoms. Among 2124 older men with minimal depressive symptoms at baseline (GDS score $\leq$ 2), those in the lowest quartile of rhythm robustness (pseudo-F statistic) were more than 2.5 times more likely to develop clinically significant depressive symptoms (GSD scores $\geq$ 6) at follow-up (OR = 2.58; 95% CI 1.11–5.59). Rhythm timing and amplitude did not significantly predict development of depression after multivariate adjustment.

Finally, using a novel non-parametric approach to analyzing activity rhythms in a cohort of 2933 older men (mean age 76 $\pm$ 5 years), Smagula et al. used latent class analysis of 24-h activity rhythms to generate subgroups with similar 24-h patterns of activity (Smagula et al. 2015). Subgroups were compared to a 'normative' category (32.1% of the cohort) that had approximately average activity representation across the 24-h spectrum, in terms of longitudinal 5-year changes in depressive symptoms. The findings suggested that timing of rhythms is a key factor related to onset of depressive symptoms. In particular, groups with later activity patterns, and certain groups with earlier activity (those with shorter active period and dampened rhythms) developed more depressive symptoms over time compared to those with normal 24-h patterns of activity. The finding that both early and late activity patterns both predicted onset of depression may suggest different etiological patterns. Specific mechanisms will require further investigation.

13.3.5 Falls and Fractures

Associations between dysregulated activity rhythm patterns and osteoporosis risk have not been thoroughly investigated. Rogers et al. (2016) examined the relationship between circadian activity rhythm patterns and risk of falls and fractures in 3001 older men form the MrOS study. After one year, 417 men (14%) had recurrent (≥ 2) falls and later acrophase (OR 1.18, 95% CI 1.06–1.32) was associated with a modestly greater likelihood of falls. After 8.6 years of >97% complete follow-up for major osteoporotic fractures, clinical spine fractures, and hip fractures, no consistent, significant associations were observed between circadian activity rhythm patterns and fractures. While later acrophase was associated with a modestly greater risk of falls, the association did not translate into a higher fracture risk suggesting that associations between dysregulated activity rhythm patterns and osteoporosis risk warrant further investigation.

13.4 Mechanisms

We consider two possibilities when interpreting these results. First, it is possible that activity rhythms directly influence morbidity and mortality in older adults independent of other features of aging. In support of this is emerging animal and human data showing the existence of both central and peripheral (e.g., in the liver, pancreas and other organs) circadian rhythms, with evidence that misalignment of internal rhythms may predispose to impaired glucose tolerance and alterations in immunological and inflammatory processes. Previous studies suggest that some but not all peripheral circadian oscillators exhibit age-related changes in rhythmicity (Yamazaki et al. 2002) and that some of these tissues retained the capacity to oscillate but were not being appropriately driven in vivo (e.g. by physical activity or feeding) (Asai et al. 2001). Indeed, previous studies suggest that some but not all peripheral circadian oscillators exhibit age-related changes in rhythmicity (Marler et al. 2006) and that some of these tissues retained the capacity to oscillate but were not being appropriately driven in vivo (e.g. by physical activity or feeding) (Kasper and Hamon 2009). The presence of arrhythmic peripheral tissues may be due to weakened behavioral and physiological rhythms that provide less effective signals to the peripheral oscillators (Lamont et al. 2007). Therefore, the change in phase relationships of behavioral and physiological rhythms may not be due to age-related changes in the entrained phase of the SCN itself but rather is because of age-related alterations in other rhythmic components of the circadian system (Marler et al. 2006). Evidence of an age-related phase advance is clear from studies involving body temperature, sleep/wake cycle, melatonin, and cortisol (Ancoli-Israel et al. 2002) in which a phase difference of about 1 h is typically found between young and old individuals. Age-related phase advances are also found in the circadian rhythms of blood pressure, iron, magnesium, neutrophils, and lymphocytes

(Van Someren et al. 1999). Acrophase deviations from the mean may represent an altered phase relationship between the circadian activity rhythm and the light dark cycle.

For example, the circadian timing system most likely affects memory, cognitive function, and behavior through a variety of neuroanatomical and neurophysiological mechanisms (Benca et al. 2009). The circadian contribution to cognition may also arise from the synchronized activities of an integrated network of clocks in the brain under the direction of the SCN pacemaker (Harmar et al. 2002). It is also possible that circadian activity rhythms are biomarkers of advanced physiological aging that provide additional risk over and beyond that of traditional covariates but which may have no direct causal association with dementia or MCI. Sleep and rhythm disturbances are common in many neurodegenerative diseases including AD (Bombois et al. 2010; Neikrug and Ancoli-Israel 2010; David et al. 2010), dementia (Loewenstein et al. 1982) and Lewy body disease (Grace et al. 2000). The major sleep complaints associated with neurodegenerative diseases include insomnia, hypersomnia, parasomnia, excessive nocturnal motor activity, sleep apnea, and sleep–wake rhythm disturbances (Chokroverty 1999). Sleep is disturbed early in the neurodegenerative process and sleep disturbances are observed in the presence of MCI (Beaulieu-Bonneau and Hudon 2009; Vitiello and Prinz 1989). Further, it has been suggested that sleep disturbance increases with severity of the neurodegeneration (Moe et al. 1995). While neurodegenerative diseases are believed to be proteinopathies resulting from excessive protein misfolding and intracellular protein aggregation, the role of sleep and rhythm disturbances in the neurodegenerative process has largely been unexplored. The accumulation of amyloid-beta in the brain extracellular space is a critical event in the pathogenesis of AD and both the sleep-wake cycle and orexin have been shown to play a role in regulating amyloid-beta dynamics (Kang et al. 2009). Orexins and their receptors are involved in a number of central (Sakurai 2007) and peripheral functions (Heinonen et al. 2008) and play an important role in maintaining wakefulness by preventing unwanted transitions into sleep as seen in narcolepsy (Saper et al. 2001).

It is also possible that circadian activity rhythms are biomarkers of advanced physiological aging that provide additional risk over and beyond that of traditional covariates but which may have no direct causal association with mortality. In this instance our data may provide evidence that circadian activity rhythms are markers for individuals with greater risk disease or death not measured by conventional markers. Although the biological mechanisms underlying the associations between disrupted activity rhythms and increased risk of CVD are unknown, the results of the Paudel et al. (2011) study suggest that the associations are independent of age, race, IADL impairments, smoking status, cognitive function, use of antidepressants, walking for exercise and history of CVD, stroke and PVD. While it is generally perceived that circadian rhythm disruptions precede CVD-related events, it is plausible that prevalent CVD disease, and/or other conditions such as diabetes, worsen circadian rhythm disruptions due to their debilitating impact on sleep/wake activity. It is also possible that circadian rhythms and conditions such as diabetes share common etiologies.

13.5 Implications

Circadian rhythms and sleep are influenced by circadian and homeostatic processes. The results of recent work in large population-based cohorts of elderly participants suggest that weak and shifted circadian activity rhythms are associated with increased risks of mortality, MCI-dementia, and CVD. Depression also appears to be associated with more desynchronized circadian activity rhythms. If these associations are confirmed in additional cohorts, future studies should examine whether interventions such as physical activity and bright light exposure that influence activity rhythms will reduce the risk of morbidity and mortality in the elderly.

For example, identifying new approaches for interventions that slow the decline in cognitive function will potentially benefit the health and well-being of the elderly before the onset of cognitive impairment. Sleep interventions and light therapy in patients or rodent models have been shown to demonstrate acute beneficial effects (Pallier et al. 2007; Wu et al. 2009). In addition, exposure to ambient light is known to influence cognition and affective state (Chaudhury and Colwell 2002; Hampp et al. 2008; Chen et al. 2008; Gonzalez and Aston-Jones 2008; Ruby et al. 2008) and bright light has phase-shifting properties that have been shown to improve cognitive performance (Campbell et al. 1995). In addition, several studies examining the efficacy of bright light for treatment of depressive symptoms in older adults without dementia have yielded promising results (Sumaya et al. 2001; Lieverse et al. 2011) although at least one has found no benefit (Loving et al. 2005). In older adults, depression has also proven difficult to treat with a high rate of treatment refractoriness utilizing first-line pharmacotherpies (Lenze et al. 2008). Meanwhile, several studies suggest bright light therapy, a chronobiological treatment designed to correct circadian rhythm disruption, may be an effective treatment for depression in older adults (Sumaya et al. 2001; Lieverse et al. 2011). Recent studies have also indicated that deterioration of the crystalline lens with advancing age decreases light transmittance and thereby, circadian photoreception (Yan and Wang 2016). Cataract surgery may improve sleep and circadian rhythms for some older adults, although there may be differences between blue light-filtering intraocular lenses compared to ultraviolet only filtering intraocular lenses. This is an area worthy of future study (Yan and Wang 2016). Overall, developing a better understanding of the relationship between circadian rhythm disruption and age-related diseases is critical and may lay the groundwork for the development of better treatment strategies.

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Chapter 14
Effects of Physical Activity on Circadian Rhythms in the Elderly

Nicolas Bessot

Abstract Aging is usually associated with a disruption of the chronobiological cycle. However, physical activity could act as a non-photic zeitgeber (external cue) for the internal clock, and thus reduce this disruption. In young participants, an effect of exercise on phase shift of circadian rhythms has sometimes been reported. A link between physical status and the circadian rhythm amplitudes has also been shown. Thus, rhythmicity alterations observed in aging could be explained by sedentary lifestyles in older adults. Few studies have explored the effects of physical activity on the circadian rhythm in the elderly. However, the biological clock seems to be enhanced in older participants with a higher level of physical capacity. The amplitude of core temperature is higher in elderly persons with a high level of aerobic capacity. A few months of physical training seems to promote circadian rhythmicity in the elderly. After training, a reduction in the fragmentation of the rest-activity rhythm, an increase in amplitude of activity-rest and core temperature rhythms, and enhancement of sleep and diurnal vigilance have been reported. We may suppose that the exercise-mediated increase in core temperature and/or melatonin and/or pupil size (increased information on light fed into the retinal-hypothalamic tract) would be the underlying zeitgeber. Another hypothesis could be that the vestibular system, when strongly stimulated by physical activity, could act as an actimeter, providing information on motion during the wake period which could then act as a zeitgeber.

Keywords Physical activity · Exercise · Training · Chronobiology · Synchronizer

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Human aging can be defined as a progressive and generalized decline. To limit the impact of the decline in ability to be independent in daily life activities, the practice of regular physical activity is indicated (WHO 2010). Aging is also associated with a disruption of the chronobiological cycle (Touitou and Haus 2000) with a deterioration in the structure of biological rhythmicity, such as temperature, sleep-wake rhythm, and alertness (Dijk et al. 2000; Hofman 2000; Van Someren 2000; Weinert 2000; Weitzman et al. 1982). Finding ways to improve the circadian timing system may therefore be useful for the well-being of elderly persons. In particular, part of the biological rhythmicity alteration observed in aging can be explained by sedentary lifestyles in older adults (Van Someren et al. 1997; Vitiello 1997). The emerging hypothesis is that physical activity acts as a non-photic zeitgeber for the internal clock. Therefore, the aim of the current chapter is to carry out a review of the effects of physical activity on circadian rhythms and to evaluate the usefulness of physical activity in counteracting the deleterious effects of aging on circadian rhythmicity.

14.1 Physical Activity as a Synchronizer of Circadian Rhythm

Various modulators of the circadian timing system, such as melatonin (Wurtman and Zhdanova 1995), bright light (Turner et al. 2010), and manipulations of body heat (Van Someren 2000) have been positively tested to resynchronize circadian rhythmicity. More recently, the hypothesis physical activity that might be a potent synchronizer of the biological clock has emerged.

There are now sufficient data from phase-shift studies to construct a phase-response curve of dim light melatonin onset compared to acute exercise in humans (Fig. 14.1). This phase-response curve includes data from studies using 1-h high intensity or 3-h lower intensity workouts on exercise machines and reveals a phase-advancing effect of exercise in the evening (before usual sleep onset and dim light melatonin onset) and a phase-delaying effect of exercise during most of the usual sleep period extending into the morning and possibly the afternoon (Baehr et al. 2003; Buxton et al. 1997, 2003; Van Reeth et al. 1994). Studies providing data used here suffered from lack of control of competing zeitgebers (e.g. light). However, the overall shape of the phase-response curve is sufficiently different from the photic curve to rule out light exposure during exercise as a likely cause of phase shifts, particularly advances in response to evening exercise and delays in response to late morning exercise (phases at which light would induce shifts in the opposite direction) (Mistlberger and Skene 2005). Effects of exercise sessions were detectable in the 1st circadian cycle after the exercise session, but not in the 2nd cycle (Buxton et al. 2003), suggesting no stable resetting of the pacemaker phase.

Since physical exercise permits a phase shift of dim light melatonin onset, some authors have tested the beneficial effect of exercise in accelerating

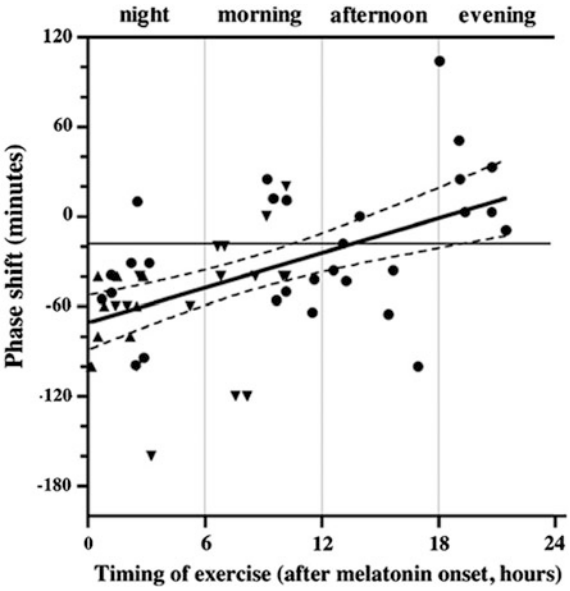

Fig. 14.1 Phase-response curve observed in response to exercise at various times of day. The magnitude of the phase shift of the circadian melatonin onset in each individual from day 1 (preexercise) to day 2 (postexercise) is plotted versus the timing of the start of exercise sessions relative to the timing of the onset of melatonin secretion preexercise. By convention, phase advances are defined as positive numbers and phase delays as negative numbers. Phase shifts in response to high-intensity, 1-h nocturnal exercise (Buxton et al. 1997) and daytime exercise are depicted by *filled circles* (Buxton et al. 2003). Phase shifts in response to low-intensity, 3-h exercise sessions from 2 previous studies are depicted by *filled triangles* (Buxton et al. 1997) and *filled downward triangles* (Van Reeth et al. 1994). Line depicts significant relationship between phase shifts and circadian time of exercise (slope significantly different from 0, $p = 0.0003$). *Dashed curves* depict 95% confidence intervals of the slope of the *line*. Modified from Buxton et al. (2003)

resynchronization after jetlag. One study appears to be particularly relevant because the authors adequately controlled for the phase-resetting effects of light on the circadian system (Barger et al. 2004). In this study, exercise in dim light conditions exhibited a significant phase shift of dim light melatonin onset of 3.17 h. However, an extended phase shift could be achieved with appropriately timed light exposure through similar protocols (Duffy et al. 1996).

The effect of acute exercise on the circadian rhythm of core temperature has been addressed in two studies which examined the interaction between exercise and bright light with circadian rhythms in humans (Baehr et al. 1999; Youngstedt et al. 2002). The combination of bright light and exercise elicited a significant phase delay, but this delay was not significantly different from that produced by bright light alone. The authors concluded that their study did not have adequate statistical power (Youngstedt et al. 2002) or lacked sufficient intensity and duration of exercise (Baehr et al. 1999).

Acute exercise studies to date have been limited primarily to young male adults. One study found no difference between young and old male and female subjects in phase-shift responses to a single 3-h bout of exercise (Baehr et al. 2003).

The investigation on chronic exercise effects by Atkinson et al. (1993) does not seem to support phase shift. Atkinson et al. (1993) compared physically active and inactive young participants. The groups did not differ with respect to phasing of the rhythms. However, to our knowledge no study has investigated the relation between the time of training and the circadian rhythms of physically active participants.

Therefore, the effect of exercise on phase shift of circadian rhythms is not very clear in humans. Applied research has suffered somewhat due to lack of control of competing zeitgebers (e.g. light), exercise characteristics (mode, intensity, and duration), and athletic status of research participants. Standardization of these factors is needed to provide evidence-based advice for use in physical activity in resynchronization protocols.

14.2 Physical Activity as an Amplifier of Circadian Rhythm

Härmä et al. (1982) reported that the rhythm amplitudes of physically fit participants were higher than in unfit individuals under standardized laboratory conditions. This study included six very physically fit (Vo2 max = 57.4 ml/kg/min) and six average fit (VO2 max = 34.6 ml/kg/min) workers (mean age 32.1). Several variables were measured at 02:00, 06:00, 10:00, 14:00, 18:00, and 22:00 h during a period of eight weeks. The main result showed that the day-night differences in several variables were more pronounced for very physically fit participants. Indeed, the very physically fit group had at least 1.5 times greater amplitude than the average group in the following variables: oral temperature, reaction time, subjective arousal, rate of perceived exertion at 3 min of exercise, and heart rate at 9 min of exercise. The estimated oral temperature amplitude in the very physically fit group was 0.41 °C, and in the average fit group it was 0.24 °C. The study by Atkinson et al. (1993) confirmed and complemented this previous study. These authors compared physically active (n = 7; age: 23.9 ± 3.3 years) and inactive young participants (n = 7; age: 24.3 ± 3.2 years). Active participants evidenced 1.5–2.5 times greater rhythm amplitudes than inactive participants for oral temperature, subjective arousal, sleepiness, flexibility, left and right grip strength, submaximal heart rate, and self-chosen work rate. The groups did not differ with respect to phasing of the rhythms, meaning that physical status mainly involves amplitude of rhythms. Individuals with high amplitudes in their circadian rhythms have been found to be more tolerant to shift work (Reinberg et al. 1980). It is thought that large amplitudes result in greater stability of circadian rhythms and that this is beneficial in coping with frequent rhythm disturbances (Fig. 14.2).

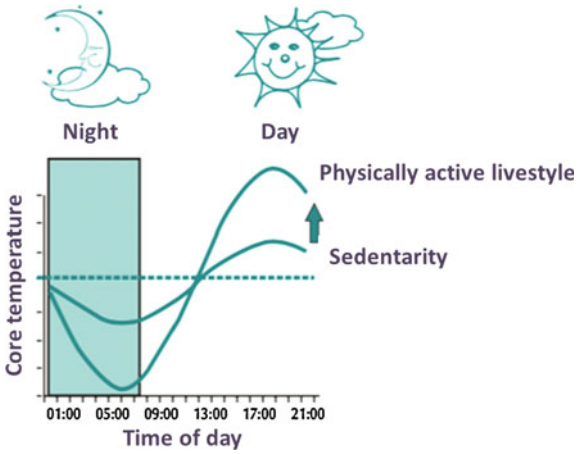

Fig. 14.2 Schematic representation of physical activity status and circadian rhythmicity of core temperature. Adapted from Davenne (2005)

These observations were also confirmed for core temperature with middle-aged shift workers (Mauvieux et al. 2003). In this study, authors used a specific methodology to confirm the stability and persistent quality of the circadian core temperature rhythms of physically active participants. They also confirmed the higher amplitude of core temperature circadian rhythms even when activity level (measured continuously by actigraphy) was taken into account. In contrast, a decrease in physical activity, by means of forced bed rest, leads to rest-activity rhythm disorders (Campbell 1984) and to a decrease in amplitude of body temperature and hormone secretion rhythms (Vernikos-Danellis et al. 1972).

The exact mechanisms linking physical activity and amplitude of circadian rhythm are unknown. However, we may hypothesize the existence of an influence of the sleep characteristics of active subjects. Chronic exercise has been commonly found to be associated with increased slow wave sleep, total sleep time, decreased rapid eye movement sleep, and sleep onset latency (Kubitz et al. 1996). Indeed, moderate aerobic exercise training has been prescribed as a pertinent non-pharmacological treatment for sleep disorders (Chennaoui et al. 2015). An improvement in physical status could be easily obtained by regular practice of physical activity. Consequently, it seems crucial to recommend physical training in order to counteract age-related decline of biological rhythms.

14.3 The Effect of Physical Status on Circadian Rhythm in the Elderly

As we know, inactivity leads to various rest-activity rhythm disorders (Campbell 1984) and to a decrease in body temperature amplitude and hormone secretion rhythms (Vernikos-Danellis et al. 1972). In older adults, as the daily level of

physical activity progressively and continuously decreases (Milanović et al. 2013), part of the alteration in the circadian rhythmicity must be the consequence of this physical inactivity. It is well known that the level of physical activity and physical capacity are interdependent. Considering the relationship between physical activity and physical capacity (Milanović et al. 2013), alteration of the circadian rhythmicity in older adults might also be dependent on their physical aerobic capacity. One study investigated the link between aerobic capacity and circadian rhythm in older adults (Dupont Rocher et al. 2016). Three groups of participants were established based on their peak oxygen consumption (Group 1 < 20 mL min^{-1} kg^{-1}; Group 2 > 20 and <30 mL min^{-1} kg^{-1}; Group 3 > 30 mL min^{-1} kg^{-1}). Each participant had an individual evaluation of circadian rhythmicity characteristics based on circadian rhythms of core temperature. Nocturnal sleep and diurnal activity were also recorded using actigraphy, and diurnal vigilance was tested using a simulated driving task. The main results are presented in Table 14.1.

An important result of this study is that the amplitude of temperature rhythm depends on the level of aerobic capacity in older participants. Van Someren et al. (1997) had already reported a trend ($p = 0.06$) for correlation between aerobic power and amplitude of the rest-activity rhythm of elderly persons. Usually, a decrease in amplitudes of different circadian rhythms, such as that of core temperature, is observed with aging. This study shows that the oral temperature amplitude for Group 3 (high aerobic capacity) is significantly higher than the oral temperature amplitude recorded for Group 1. In addition, Group 2 (intermediate aerobic capacity) and Group 3 were significantly more active during the day than Group 1. The greater amplitude of the core temperature rhythm observed in Group 3 may have been due to motor activity during the day which was also greater,

Table 14.1 Effect of physical status on oral temperature rhythm, activity, and diurnal vigilance (driving performance) (Mean ± SD) (adapted from Dupont Rocher et al. 2016)

		Group 1 (low)	Group 2 (intermediate)	Group 3 (high)
$V02_{peak}$ ($mL.min^{-1}.kg^{-1}$)		**17.6 ± 2.2**	**25.3 ± 4***	**35.4 ± 3.9*,[a]**
Oral temperature rhythm	Mesor (°C)	36.75 ± 0.63	36.43 ± 0.34	36.90 ± 0.47
	Amplitude (°C)	**0.43 ± 0.09**	**0.69 ± 0.34**	**0.83 ± 0.32***
	Phase (h:min)	15:15 ± 05:40	15:17 ± 03.44	16:10 ± 03.58
Actigraphy variables	Diurnal motor activity ($Mvts\ min^{-1}$)	**13.1 ± 7.2**	**31.4 ± 13.7***	**28 ± 11.4***
	Index of night inactivity (%)	**81.9 ± 15**	**93.8 ± 2.3***	**91 ± 7.1***
Driving performance	Number of lane crossings	**15.3 ± 21.4**	**2.7 ± 2.9***	**2.3 ± 3.5***
	SD of lateral position	0.49 ± 0.16	0.44 ± 0.05	0.43 ± 0.07

*Different from Group 1 ($p < 0.05$)
[a]Different from Group 2 ($p < 0.05$)
Its in bold if there is any significant group effect

resulting in what is called a "masking effect" (Hanneman 2001). However, several arguments provide a basis for rejection of this hypothesis. First, Group 2 and Group 3 have a different mean motor activity during the day, while the amplitude of core temperature rhythm is not significantly different between these two groups. Second, regardless of the group, the rhythms of activity/rest and core temperature are not in phase. Time to peak for the core temperature rhythm is longer than the activity-rest rhythm (for Group 3: 16:10 h vs. 12:37 h).

Group 2 (intermediate aerobic capacity) and Group 3 (high aerobic capacity) have higher indexes of inactivity during the night compared to Group 1. These results mean that participants with greater aerobic capacities sleep better than those with poor aerobic capacity and confirm the positive impact of physical activity on sleep quality (Van Someren et al. 1997). Concomitant with an increase in sleep quality and a reinforcement of circadian rhythms, an increase in diurnal vigilance was also observed in the groups with higher levels of physical activity. Vigilance was assessed using a simulated driving task (Davenne et al. 2012) in monotonous traffic conditions and during the post-lunch dip in vigilance. Results showed a significantly greater number of lane crossings for Group 1 compared to Groups 2 and 3.

The biological clock seems to be enhanced in older participants that have a higher level of physical capacity. Physical activity directly or indirectly impacts the circadian clock, which is the major vector of biological rhythm, and thus fights against circadian clock aging effects. However, aerobic capacity could also be a marker of overall health status, and the youth of the biological rhythm observed for high aerobic capacity groups could be dependent on the overall health status of participants. In order to partly overcome this bias, studies that included a physical training protocol have been conducted in the elderly and are described below.

14.4 Effects of Physical Training on Circadian Rhythms in the Elderly

The effect of increased physical fitness on circadian rhythms has rarely been investigated previously in elderly participants. The study conducted by Van Someren et al. (1997) investigated whether the circadian rest-activity rhythm in healthy elderly persons improve after long-term fitness training. For this purpose, healthy elderly males participated in a 3-month training program. Fitness training induced a significant reduction in the fragmentation of the rest-activity rhythm after training. It is important to note that none of the participants in this study declared lifestyle changes with regard to the general engagement in activities or time spent outdoors. Thus, effects of other external synchronizers such as light, could not explain this result. Nevertheless, Van Someren et al. (1997) did not report any effect on the amplitude of the activity-rest rhythm, while Mauvieux et al. (2002) reported a significant effect of 4 months of physical training on the amplitude of activity-rest

and core temperature rhythms. These observations are supported by the fact that exercise training in elderly persons may enhance sleep (Montgomery and Dennis 2004) and diurnal vigilance (Gruau et al. 2003).

Most recently, an experiment was conducted in a group of 24 participants (>60 years old) randomly assigned to a training group or a control group (Dupont Rocher 2009). A multivariate training program (aerobic and strength exercises) was carried out for 16 weeks in the trained group. Results did not reveal any effects of this training program on actigraphy, which is contradictory to previous reports (Mauvieux et al. 2002; Van Someren et al. 1997). However, the study of Dupont Rocher (2009) confirmed the effect of physical training on the amplitude of the circadian rhythm of core temperature (Fig. 14.3) as reported previously (Mauvieux et al. 2002). A new PhD thesis recently completed in our team specifically evaluated the effect of two training methods on circadian rhythms in the elderly (Bigot 2017). The first method of training was traditional, with a coach coming directly to the participants' homes, and the second method used a video conference allowing the coach to stay at home and train a few elderly persons at the same time. A control group was added to control for seasonal effect. The results of this PhD thesis confirms the effect of training (regardless of the method) on the circadian rhythm of activity-rest. As expected, the amplitude and mesor of the activity-rest rhythm were

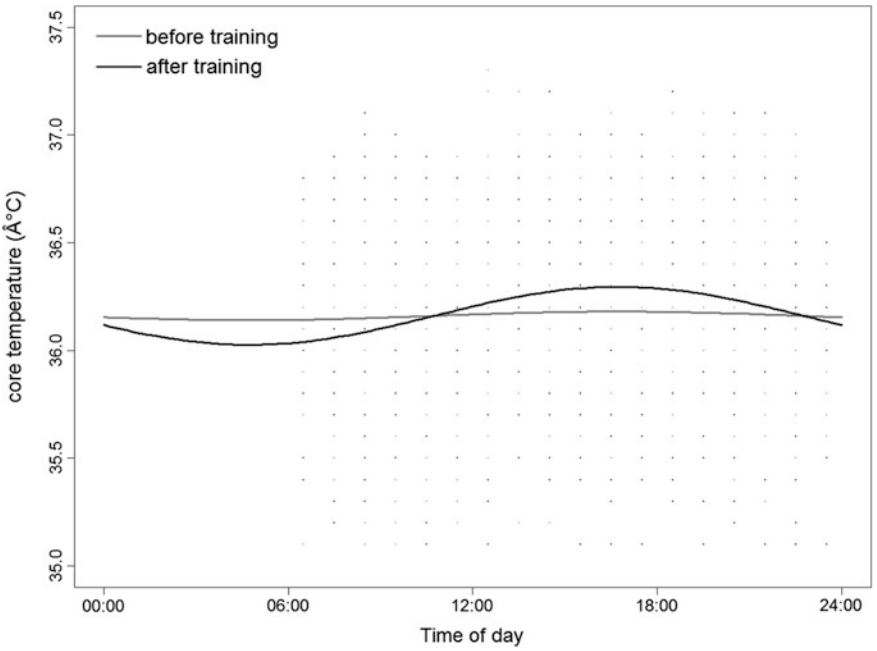

Fig. 14.3 Circadian rhythm of oral temperature in trained participants. Points represent raw data and lines represent COSINOR adjustments to the data (Dupont Rocher 2009)

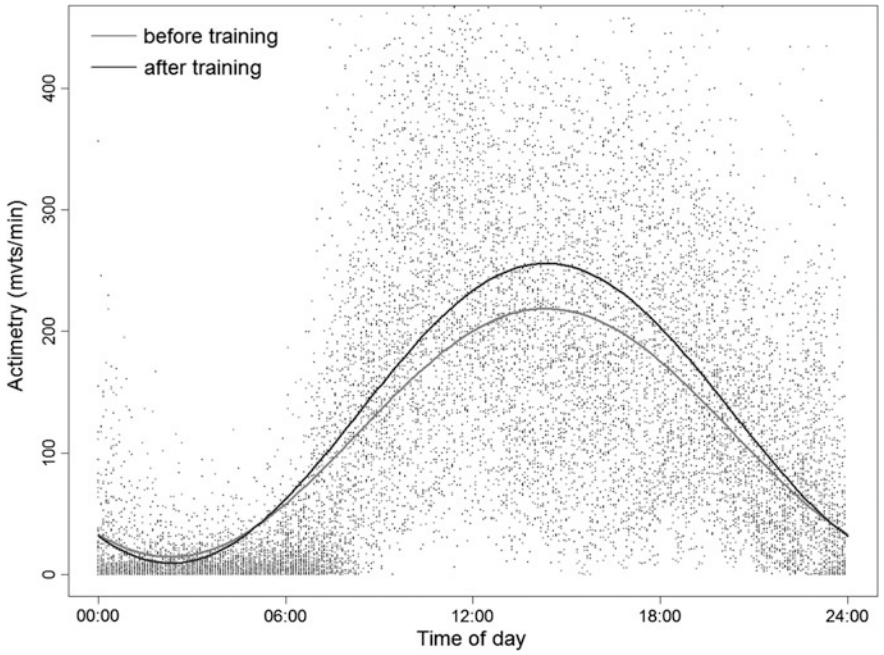

Fig. 14.4 Activity-rest rhythm of trained participants (training with video conferencing or at home coaching). *Points* represent raw data and *lines* represent COSINOR adjustment to the data (Bigot 2017)

increased after both types of training (Fig. 14.4). In this study, no effect was observed on core temperature rhythm.

In all studies described above, only small groups of participants were evaluated, reducing the statistical power of the analysis. This could explain the lack of consistency in the reported results. Although further investigations are needed, the hypothesis of a positive effect of physical training on circadian rhythmicity seems to have validity at this time.

14.5 How Does It Work?

The hypothesis that physical activity acting as a non-photic zeitgeber (external cue) for the internal clock has become increasingly popular (Escames et al. 2012). However, there is no clear answer as to whether this links physical activity to internal clock function.

In rodents, there is evidence that the central nervous system cells receive information related to the activity-rest state of the organism via afferent signals from the lateral geniculate nucleus (Redlin and Mrosovsky 1997). Both serotonergic and adrenergic inputs to the circadian system from the midbrain raphe nuclei and the locus coeruleus, respectively, have also been implicated in mediating the effects of activity on the circadian clock in rodents (Strüder and Weicker 2001).

Given the fact that exercise-mediated changes, such as electroencephalographic activity during sleep, are absent when the body is cooled (Horne and Moore 1985), it is possible that an exercise-mediated increase in core temperature would be the underlying zeitgeber. Van Someren (2003) provided a detailed account of how the biological clock is sensitive to local brain temperature as well as skin temperature. Zeitgeber effects of temperature were thought to be less pronounced than those of light, but no less important in terms of usefulness.

Another hypothesis could be that physical activity effects on the internal clock is due to melatonin. Moderate or strenuous exercise seems to increase nocturnal plasma melatonin in humans (Knight et al. 2005). However, some contradictory results have been reported (Atkinson et al. 2003). If the effects of exercise on melatonin can be clarified through careful control of time of exercise and light conditions, then it will be possible to postulate a causal nexus.

Exercise could possibly mediate a number of ocular effects (Harbin et al. 1989), perhaps including an increase in pupil size of the retina. Greater intensities and durations of exercise have been associated with larger pupil size (Ishigaki et al. 1991). Consequently, a possible indirect effect of exercise could be increased information on light provided to the retinal-hypothalamic tract. This tract has been linked with light-induced suppression of plasma melatonin (Gaddy et al. 1993).

The vestibular system might also be a synchronizer of the biological clock. The vestibular system, which detects every type of movement, may be involved in physical activity effects on the internal clock.

Recently, the involvement of the vestibular system in the regulation of circadian rhythmicity in animal models has emerged, including a direct influence on the free-running circadian period (Fuller and Fuller 2006). In one study, total suppression of vestibular input in rats led to a sharp fall in core temperature and a switch to an ultradian rhythm in the days following vestibular lesion (Martin et al. 2015). Vestibular inputs seem to be involved in daily rhythm regulation by influencing the CNS independently of direct proprioceptive and muscular inputs. A lack of synchronization between temperature and rest-activity cycles has also been observed in humans with bilateral vestibular loss (Martin et al. 2016). This result supports the hypothesis that vestibular inputs are salient features of the circadian clock and enhance the stabilization and precision of both external and internal entrainment. In particular, the emerging hypothesis is that the vestibular system could normally act as an actimeter, providing information about motion during the wake period.

14.6 Conclusion

Aging is associated with a disruption of the chronobiological cycle (Hofman 2000; Weinert 2000). In addition, part of the biological rhythmicity alterations observed in aging can be explained by sedentary lifestyles of older adults (Van Someren et al. 1997; Vitiello 1997). The hypothesis developed in the current chapter is that physical activity could act as a non-photic zeitgeber (external cue) for the internal clock. The positive effects of physical activity on circadian rhythmicity are demonstrated, although results are still inconsistent. However, most studies described here have suffered somewhat from lack of control of competing zeitgebers (e.g. light), exercise characteristics (mode, intensity, and duration), and athletic status of research participants. Therefore, standardization of these factors is needed for evidence-based advice to be provided for use in physical activity programs. In addition, physical activity and light could be two disconnected synchronizers and thus have a cumulative impact on the biological circadian clock in combatting the effect of aging. Drawing a clear distinction between light and physical activity effects on the circadian clock thus requires further investigation.

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